Variation of the *PALB2* and *PTEN* Genes and eIF4E Protein Expression as Prognostic Factors in Breast Cancer

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List of Original Publications

This thesis is based on the following three original publications, which are referred to in the text by their Roman numerals:

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II. Heikkinen T, Greco D, Pelttari LM, Tommiska J, Vahteristo P, Heikkilä P, Blomqvist C, Aittomäki K, Nevanlinna H. Variants on the promoter region of PTEN affect breast cancer progression and patient survival. Breast Cancer Res 2011;13:R130.

III. Heikkinen T, Korpela T, Fagerholm R, Khan S, Aittomäki K, Heikkilä P, Blomqvist C, Carpén O, Nevanlinna H. Eukaryotic translation initiation factor 4E (eIF4E) expression is associated with breast cancer tumor phenotype and predicts breast cancer survival after anthracycline chemotherapy treatment. *Submitted*.

Abbreviations

Gene names are written in *italics*.

4EBP	Eukaryotic translation initiation factor 4E binding protein
aa	Amino acid(s)
A	Adenine
AKT	Homolog for V-akt murine thymoma viral oncogene
AT	Ataxia-telengiactasia
ATM	*Ataxia-telengiactasia mutated*
ATR	Ataxia-telengiactasia and Rad3 related
BACH1	*BRCA1-associated C-terminal helicase-1*
BAMBI	*BMP and Activin Membrane-Bound Inhibitor*
BER	Base excision repair
BLM	*Bloom syndrome, RecQ helicase-like*
BRCA1	*Breast cancer gene 1*
BRCA2	*Breast cancer gene 2*
BRCT	BRCA1 carboxy-terminal repeat
BRIP1	BRCA1 interacting protein 1
C	Cytosine
C10orf11	*Chromosome 10 open reading frame 11*
Casp8	*Caspase 8*
CBF-1	C-promoter binding factor-1
CDH1	*Cadherin 1*
CEF	Cyclophosphamide, epirubicin, and fluorouracil polychemotherapy
CHK1/CHEK1	Checkpoint kinase 1
CHK2/CHEK2	Checkpoint kinase 2
CI	Confidence intervall
CISH	Chromogenic in situ hybridization
CK	Cytokeratine
CMF	Cyclophosphamide, methotrexate, and fluorouracil polychemotherapy
CSGE	Conformation sensitive gel electrophosresis
C-teminal	Carboxy-terminal
D, Asp	Aspartic acid
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
Egr-1	Early growth response protein 1
eIF4A	Eukaryotic translation initiation factor 4A
eIF4E	Eukaryotic translation initiation factor 4E
eIF4F	Eukaryotic translation initiation factor 4F complex
eIF4G	Eukaryotic translation initiation factor 4G
ER	Estrogen receptor

ERBB2	*Avian erythroblastic leukemia viral oncogene homolog 2*
FA	Fanconi anemia
FANCA/B/C/D1/D2/E/F/G/J/L/M/N/O/P	Fanconi anemia genes (complementation groups)
FGFR2	*Fibroblast growth factor receptor 2*
G	Guanine
GEO	Gene Expression Omnibus
GWAS	Genome-wide association study
H, His	Histidine
H2AX	H2A histone family member x
HER2	Human epidermal growth factor receptor 2
HR	Homologous recombination
HR	Hazard ratio
IHC	Immuno histochemistry
L, Leu	Leusine
LD	Linkage disequilibrium
LKB1/STK11	*Serine threonine kinase 11*
LSP1	*Lymphocyte-specific protein 1*
M	Distant metastasis
MAP3K1	*Mitogen-activated protein kinase kinase kinase 1*
MAPK	Mitogen-activated protein kinase
MCL-1	Myeloid Cell Leukemia-1
MDC1	Mediator of DNA damage checkpoint protein 1
miRNA	Micro RNA
MLPA	Multiplex ligation-dependent probe amplification
MMP-9	Matrix metalloprotease 9
MMR	Mismatch repair
MORF4L1/MRG15	*Mortality factor 4 like 1*
MRE11	Homolog for Saccharomyces cerevisae meiotic recombination 11
mRNA	Messenger RNA
mTOR	Mechanistic target of rapamycin
MYC	*Homolog for avian v-myc myelocytomatosis viral oncogene*
N	Nodal metastasis
NBS1	Nijmegen breakage syndrome 1
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NMR complex	NBS1, MRE11, RAD50 complex
NQO1	*NAD(P)H dehydrogenase, quinone 1*
N-terminal	Amino-terminal
OCA2	*Oculocutaneous albinism II*
OR	Odds ratio
p	Short arma of a chromosome
P, Pro	Proline
PALB2	*Partner and localizer of BRCA2*
PCR	Polymerase chain reaction
PDK1	Phosphoinositide dependent kinase 1

PgR	Progesterone receptor
PI3K	Phosphatidylinositol kinase
PIK3CA	*Phosphatidylinositol kinase alpha*
PIK3CB	*Phosphatidylinositol kinase beta*
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PJS	Peutz Jeghers syndrome
PTEN	*Phosphatase and tensin homolog*
PTENP1	*Phosphatase and tensin homolog pseudogene 1*
PTHS	*PTEN* related hamartoma syndrome
PTT	Protein truncation test
q	long arm of a chromosome
RAD50	Homolog for Saccharomyces cerevisae Rad50
RAD51	homolog for Saccharomyces cerevisae Rad51
RAD51C	Homolog for Saccharomyces cerevisae Rad51 C
RAD51D	Homolog for Saccharomyces cerevisae Rad51 D
RAD51L1/RAD51B	Homolog for Saccharomyces cerevisae Rad51 B
RAP80	Receptor associated protein, 80-KD
RNA	Ribonucleic acid
RNF8	Ring finger protein 8
S, Ser	Serine threonine kinase 11
SLX4	*Homolog for Saccharomyces cerevisae Structure-specific endonuclease subunit SLX4*
SNP	Single nucleotide polymorphism
Sp1	Sp1 transcription factor
SSA	Single strand annealing
T	Tumor size
T	thymine
TGFB1	*Transforming growth factor, beta 1*
TLK1B	Tousled-like kinase 1B
TMA	Tissue micro array
TOPBP1	Topoisomerase II binding protein 1
TOX3/TNRC9	*TOX high mobility group box family member 3*
TP53	*Tumor protein p53*
TUBB2C	Tubulin, beta 2B class IIb
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
XRCC2	*X-ray repair complementing defective repair in Chinese hamster cells 2*

Abstract

Breast cancer is the most common cancer in women and the leading cause of female cancer mortality. Family history of the disease is one of the major risk factors for breast cancer, and the hereditary factors also play a role in breast cancer survival. Investigation of the inherited variants for breast cancer risk, tumor phenotype, and prognosis can be beneficial for further improvements of clinical management of breast cancer patients. Recognition of patient groups with poor prognosis remains one of the major challenges in cancer research.

The aim of this work was (I) to study the association of the *PALB2* 1592delT Finnish founder mutation with breast cancer risk, and together with *BRCA1* and *BRCA2* mutations on tumor phenotype and patient survival; (II) to investigate the role of germline *PTEN* promoter variation on breast cancer risk, tumor phenotype and patient survival, and (III) to evaluate the association of eIF4E protein expression with characteristics of breast tumors as well as on patient survival and treatment outcome. The germline variants were studied in a set of DNA samples from 1,870 unselected and 542 additional familial breast cancer patients with detailed information on tumor characteristics and patient survival, and from 1,079 population controls. The eIF4E protein expression was studied on tissue microarrays with 1,423 breast tumors. RNA expression analysis using gene expression microarrays was carried out in 183 fresh frozen tumor tissue samples.

The *PALB2* gene is a moderate-penetrance breast cancer susceptibility gene with approximately fourfold increased risk for the carriers of a truncating mutation. The 1592delT mutation was present in 19 familial (2.0%) and 8 sporadic (0.6%) breast cancer patients and in 2 (0.2%) population controls. This confirmed the risk effect of the mutation and demonstrated a trend of increasing frequency of the mutation with increasing family history of breast cancer, with a highly increased risk for familial breast cancer. The tumors of the *PALB2* mutation carriers were more often of higher grade, expressed higher levels of Ki67 proliferation marker and were more frequently of the clinically challenging triple negative subtype. The *PALB2* mutation associated with survival of the familial breast cancer patients particularly among HER2 negative cases. An even stronger survival effect was seen in patients carrying a *BRCA2* mutation, but not among *BRCA1* mutation carriers.

The PTEN protein is a negative regulator of the PI3K/Akt oncogenic pathway. Inherited inactivating mutations in *PTEN* cause PTEN hamartomatous polyposis syndromes (PHTS) including Cowden syndrome, in which the estimated risk for breast cancer is 25-50%. The promoter region in 330 familial breast cancer patients was analyzed for germline genetic variation and the genotypes of three low-frequency promoter polymorphisms (-903GA, -975GC, and -1026CA) were further determined in 2412 breast cancer patients. All three variants significantly associated with decreased survival. The gene expression profiles of the breast tumors of promoter variant carriers and non-carriers identified a signature of 160 differentially expressed genes. The expression of these genes stratified patients into two groups with distinct survival patterns supporting the effects of germline variants on gene expression signatures and on metastatic development in breast cancer.

Abnormal translation is a mechanism that frequently occurs during carcinogenesis. A major regulator of translation of many cancer-related transcripts is mammalian translation initiation factor eIF4E, often overexpressed in various cancers and associated with poor prognosis in breast cancer. Here, the eIF4E protein expression was analyzed in breast tumor tissue samples using immunohistochemistry. The expression of eIF4E was associated with multiple characteristics of aggressive breast cancer and with the triple negative subtype. High expression of eIF4E also associated with the survival of breast cancer patients and in subgroup analysis a particularly strong effect was identified in patients treated with anthracycline-based chemotherapy, whereas in the non-chemotherapy group there was no significant survival difference. This is, to our knowledge, the first time eIF4E has been associated with treatment outcome in a clinical dataset. These results support eIF4E as a prognostic and predictive marker and emphasize the potential of therapies targeting this protein.

Identification of the effects of breast cancer risk mutations on tumor phenotype and patient survival can have direct impact on the clinical management of the patients harboring these mutations. These patients could benefit from extended and more intense follow-up and more aggressive treatment, or preventive measures. The knowledge about the causative genes and the genetic variants associated with increased cancer risk, tumor phenotype, and prognosis also provide new insights into breast cancer tumorigenesis.

1 Introduction

Breast cancer is the most common cancer in women and family history is among the major risk factors of this complex disease, with first degree relatives of a breast cancer patient having approximately two-fold increased risk of the disease compared to the general population. Inherited factors are also involved in sporadic cases with estimations of having some effect in approximately 30% of all breast cancer. Germline variants in multiple loci have been associated with breast cancer affecting the disease risk with varying penetrance. Rare high-penetrance susceptibility mutations mainly in the *BRCA1* and *BRCA2* genes substantially increase the life-time risk of developing breast cancer. In addition to high-penetrance mutations, variants affecting breast cancer risk with moderate or low increase in risk have also been identified. The high-penetrance variants are typically rare and lower penetrance variants more common in the population. Founder effects of the susceptibility mutations are also frequent in certain populations, for example in Finland.

The prognosis of breast cancer is affected by various factors related to the biological properties of the tumor. Numerous prognostic biomarkers have been identified, providing information on the outcome of the disease and some also with predictive value by predicting the impact of a treatment, such as the expression of estrogen receptor (ER) or HER2, which make the tumors responsive for endocrine and anti-HER2 antibody treatments, respectively. A need for novel biomarkers remains and they may in the future help to further improve the outcome of breast cancer patients. The prognosis of breast cancer also has a heritable component but the exact molecular mechanisms have remained largely unknown. The plausible mechanisms include for instance the effects of the germline variants on the biological properties of tumors or effects carried out through pharmacogenetic functions.

This study investigates the associations of the Finnish founder mutation 1592delT in the moderate-penetrance breast cancer susceptibility gene *PALB2* and germline promoter variants of the *PTEN* gene with breast cancer risk, tumor features and patient survival. The study also looks into the associations of the translation initiation factor eIF4E protein expression with breast cancer tumor features and prognosis with treatment stratified survival analyses.

2 Review of the Literature

2.1 Cancer

2.1.1 Biology of cancer

Cells grow, divide, and die under the control of tissue specific regulatory signaling networks adjusted by the developmental phase of the organism and the surrounding environmental conditions. A cell may escape this control and begin to proliferate with an increased rate to form a tumor, and eventually with further malignant transformation, cancer. The development of a tumor follows the principals of Darwinian natural selection. The changes in DNA introduce cancer cells with adaptations to the environment, and these changes are then inherited to the offspring of the cell. In a monoclonal tumor all cells are descendants of the cell in which the changes for malignant transformation have occurred (Nowell 1976). Through tumor evolution, the tumor cells can adapt to changes affecting their growth, such as cancer treatments. A developed tumor may consist of cells of distinct types carrying out different functions required for malignant growth, which together with metastatic cells the tumor may have released to invade other parts of the body, originate from a common ancestor, but may have further developed in different branches of the evolutional tree and eventually bear little similarity (Collisson et al. 2012).

For a tumor to transform into cancer it must develop an ability to proliferate without external growth signals from surrounding cells and other tissues, to tolerate growth inhibitory signals, and to evade apoptosis. It must develop an unlimited growth potential by preventing the shortening of telomers during cell division. When the tumor is growing in size it must secure the access of oxygen and nutrition by sustaining angiogenesis. Eventually the tumor develops an ability to enter neighboring tissues and to send metastases to other parts of the body. These classical hallmarks of cancer (Hanahan, Weinberg 2000) are followed by two new hallmarks of reprogramming energy metabolism and evading destruction by the immune system (Hanahan, Weinberg 2011). The process to acquire these properties requires changes in multiple pathways through mutations and regulatory changes affecting genes in multiple loci. This is often made possible, together with inflammation, by increased mutational rate and genomic instability of the tumor cells, caused by the changes in the genes controlling genetic integrity.

DNA is under constant pressure to change by damage caused by endogenous metabolic products and external DNA-damaging agents such as oxygen radicals, gamma or ultra violet radiation, and mutagenic chemicals. DNA changes can be single-nucleotide changes, small or large insertions and deletions, chromosomal translocations, inversions, or changes in the overall chromosome number. To prevent accumulation of mutations and to maintain genomic integrity, cells possess a wide range of DNA-repair mechanisms. Among the most harmful DNA-damages to cell are double strand breaks, which can be repaired with high accuracy by using the undamaged strand as a template in the process called homologous recombination (HR) DNA repair. Other important double-strand break repair mechanisms are non-homologous end joining (NHEJ) and single strand annealing (SSA), which are less accurate than HR and result in a higher frequency of nucleotide changes in the repaired site. DNA single strand breaks can be repaired with the mechanism of base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR) (Madhusudan, Middleton 2005). Alterations in these pathways can lead to an increased mutation rate and further mutations in other cancer genes. In addition to mutations changing the DNA sequence, epigenetic changes, such as hypermetylation of CpG islands in the regulatory regions of the genes, are frequent in affecting the expression of cancer genes in tumors.

Cancer can originate basically in any type of human tissue. Depending on the type of tissue where the cancer originates, the tumors are classified into different categories. Carcinomas, for example, originate from epithelial tissues, sarcomas from mesenchymal connective tissue, lymphomas from lymphocytes, and leukemias from bone marrow. The different cancers vary, however, greatly in their biology, incidence, and outcome. Of the globally estimated number of 12.7 million yearly cancer cases, the most common cancer in men is lung cancer and in women breast cancer (Jemal et al. 2011). The difference in cancer incidence between economically developed and developing countries are large with developed countries having higher cancer-incidence rates particularly in cancers related to life-style factors, such as smoking, physical inactivity, and diet. In developing countries, cancers related to infections, such as stomach and liver cancers associated with *Helicobacter pylori* and hepatitis B and C virus infections, respectively, are more frequent (Jemal et al. 2011).

Annually 7.6 million people die of cancer (Jemal et al. 2011). The vast majority (90%) of the cancer deaths are the result of metastases that the primary tumor has released to invade distant tissues in other parts of the body. The biological mechanisms of metastasis are not thoroughly

understood, but the process can be organized into two phases: "(i) physical translocation of a cancer cell from the primary tumor to the microenvironment of a distant tissue and then (ii) colonization" (Chaffer, Weinberg 2011). To be able to form a metastatic tumor in a distant site, a population of cells needs to develop an invasive phenotype, leave the primary tumor, invade the surrounding tissues, and access circulation. Circulating tumor cells must then transit to a distant organ and exit the circulation. They need to evade the immune response of the new host tissue and survive as a single cell or as a small cell cluster and finally adapt to the new environment and begin to proliferate (Chaffer, Weinberg 2011). Malignancies originating in different sites have distinct prognosis with great variation. Cancers in tissues like the pancreas or ovaries have in general very poor prognosis since they are often detected at a late stage and have had time to develop into a more invasive form, whereas tumors arising in more accessible tissues are more often detected in time (Siegel et al. 2012). The identification of patient groups with poor prognosis, for instance due to aggressive properties of the tumor or weak treatment response, remains one of the major challenges in cancer research.

2.1.2 Cancer genes and mutations

The cancer genes are genes in which changes in function or expression can lead to cancer development. Cancer genes are traditionally classified into oncogenes and tumor suppressor genes. Oncogenes act in normal cells as proto-oncogenes having functions mainly in regulation of growth, apoptosis, proliferation, and differentiation of cells. Activating mutations in proto-oncogenes introduce them with tumor promoting functions. Oncogenic mutations affect the tumor dominantly so that the other allele of the gene does not need to be changed. These mutations are typically gain-of-function mutations that introduce new functions to the gene or cause them to be constantly in active conformation and carry out their function out of the control of the regulatory mechanism of gene expression (Varmus 1984). The oncogenes are also potential therapeutic targets, and inactivation of even a single oncogene is often sufficient to impair cancer cell growth and survival, which is refered to as oncogene addiction (Weinstein 2002).

The other class of cancer genes is tumor suppressor genes, which inhibit the formation of tumors. Mutations in tumor suppressors affect the tumor recessively and mutations in them are in general inactivating loss of function mutations (Weinberg 2007). Tumor suppressors are further classified into two main categories of gatekeeper and caretaker genes (Kinzler,

Vogelstein 1997). Of the two, gate keepers are the classical tumor suppressors and they regulate the tumor growth directly, for example by regulating cell division, differentiation, and apoptosis. Caretaker genes prevent tumor formation by maintaining genomic integrity through DNA damage response. Inactivating mutations in these genes lead to an increased mutation rate which can cause mutations in, for example, gatekeeper genes and in oncogenes. A third class of tumor suppressors are landscapers, which affect tumorigenesis outside the tumor from neighboring tissues through intercellular effects (Kinzler, Vogelstein 1998).

Massively parallel sequencing approaches have recently been applied to produce detailed genomic information on various cancers. Tumors typically harbor few driver mutations, positively selected in tumor evolution, which causally affect oncogenesis by introducing the cancer cell with a growth advantage. A driver mutation is not necessarily required for the maintenance of the tumor state, but it has given the tumor the growth advantage at least in one point of its evolution. In addition to driver mutations tumors also harbor from tens to more than 100,000 unselected, more or less randomly distributed, passenger mutations arising from the increased mutation rate. Passenger mutations do not contribute to tumor development, but have not been selected out as harmful for the tumor cell either (Stratton et al. 2009). The identification of driver mutations from passengers remains a challenge, as remains the identification of causal variants in genetics of other traits as well. It is also possible that some passenger variants affect the responsiveness of a tumor for a certain treatment, making them potential therapeutic targets (Muller et al. 2012).

The overall number of mutations varies in different cancers. Relatively low numbers are typical to cancers occurring at childhood or at early adulthood. A low mutation rate is characteristic in medulloblastomas, testicular germ cell tumors, acute leukemias, and in carcinoids. A very high number of mutations is typical in cancers which arise due to strong mutagenic exposure, such as melanomas affected by ultraviolet light exposure, lung cancer affected by tobacco carcinogens, and also in cancers with DNA repair defects, such as colorectal or stomach cancers with defective DNA mismatch repair (Stratton 2011, Collisson et al. 2012). In addition to mutations, epigenetic changes can drive tumor development through mechanisms such as hypermethylation of CpG islands, and chemical modification of histones (Esteller 2011a) and through non-coding RNAs (Esteller 2011b), but so far they have not been characterized nearly as thoroughly as sequence changes.

2.1.3 Hereditary predisposition to cancer

Family history of the disease is a major risk factor in various cancers and a strong familial clustering of cancer cases is often seen among patients. Hereditary cancer, however, only accounts for a small proportion of overall cancer incidence, whereas environmental factors are the main causative factor (Lichtenstein et al. 2000). In the families with highly increased risk for cancer, genetic knowledge can, nevertheless, be of crucial importance by helping to identify the tumor at an early stage, and hence reducing the mortality rate of the disease. Hereditary cancers tend to occur at an earlier age with multiple primary tumors and definitive biological properties (Foulkes 2008). Hereditary factors also play a role in sporadic cancer, but do account for a considerably smaller proportion of the overall risk than in cancer families. In sporadic cancer the hereditary factors consist of common low-penetrance variants, typically increasing the risk by less than 1.5 fold. An increasing number of these common polymorphisms have been identified with genome-wide association studies (GWAS) in many common cancers with massive sample sets of tens of thousands of cases and controls (Fletcher, Houlston 2010). Mutations in high-penetrance genes are typically rare, as they would be selected out in evolution and can sometimes be lethal when homozygous. Identified low-penetrance alleles have been common in the population studied, and although rare low-penetrance alleles are likely to exist, they are extremely difficult to study as their detection would require a study with a massive sample size. Between the common low-penetrance variants and the rare high-penetrance mutations, identified in high-risk cancer families only, are moderate-penetrance variants which in general are enriched in cancer families, but are also found in sporadic cancer patients and at a low level in unaffected individuals of the general population (Foulkes 2008, Fletcher, Houlston 2010).

In hereditary cancer, mutations in cancer genes are inherited as germline variants which are present in all of the cells of an individual, extensively increasing the risk of developing the cancers related to those mutations. The hereditary cancer mutations occur nearly always in tumor-suppressor genes and hardly ever in oncogenes, as germline oncogene mutations are likely to be embryonically lethal (Frank 2001). According to the classical two-hit hypothesis, both alleles of a tumor suppressor gene need to be inactivated to induce tumor development (Knudson 1971). This is more likely to happen in an individual's lifetime, if one allele is already mutated as a germline variant, than if both alleles would need to be inactivated. Hence

the tumor suppressor mutations function on the cellular level recessively, but on the germline level, often dominantly.

The wild-type allele of a mutated cancer gene can be lost with different mechanisms of loss-of-heterozygosity, for example through deletion of the region, with inactivation through a second mutation, or through epigenetic silencing (Foulkes 2008). In addition to the classical two-hit hypothesis sometimes only one hit in a tumor suppressor is sufficient for tumorigenesis. In the case of haploinsufficiency, the other allele of a gene is inactivated through mutation and the other functional allele alone is not enough to carry out the function of the gene. Haploinsufficiency can be complete or partial and it can also be dependent on tissue type, developmental state, and environmental factors. In dominantly negative cases, a mutated allele affects the function of the protein product of the normal allele, accumulating the deleterious effect of the mutation.

2.2 Breast cancer

2.2.1 Biology and epidemiology

Breast cancer is the most common cancer in women worldwide with 1.38 million cases diagnosed yearly, accounting for 23% of the new cancer cases. It is also the leading cause of female cancer mortality, with 458,400 deaths in 2008 (Jemal et al. 2011). In Finland, 4,144 women were on average diagnosed with breast cancer annually in 2006-2010 and 848 women died of breast cancer. Breast cancer is also found in men, but is very rare with approximately 20 cases yearly in Finland (Engholm et al. 2012). Breast cancer is a complex disease with multiple known risk factors, many of which are related to the breast tissues' exposure to the hormone estrogen. These are early age of menarche, late age at menopause, a low number of pregnancies, a short-term of breast feeding, and use of post-menopausal hormone replacement therapies (Fasching et al. 2011). Every year younger age at menarche increases the breast cancer risk by a factor of 1.05, and every year older at menopause increases risk by a factor 1.03 (Collaborative Group on Hormonal Factors in Breast Cancer 2012). The risk decreases by 7% for every child birth, and by 4% for every 12 months of breast feeding (Collaborative Group on Hormonal Factors in Breast Cancer 2002). Other risk factors include mammographic density, postmenopausal obesity, and alcohol consumption (Fasching et al. 2011). Family history of the disease is also a major risk factor, with the risk compared to women with no affected first degree relatives increasing by a factor of 1.80 for one, 2.93 for

two, and 3.90 for three or more first degree relatives with breast cancer (Collaborative Group on Hormonal Factors in Breast Cancer 2001).

The human breast consists of fat, connective tissue, and glands that are divided to lobes, which are connected towards the nipple by milk ducts. Breast cancers originate from the breast epithelium, classifying them as carcinomas. Breast cancers are further classified as ductal, if they resemble the epithelial cells lining the milk ducts, and lobular if they resemble the cells of the breast lobules. Ductal carcinoma is the most common type, accounting for 70-80% of cases, and lobular accounting for approximately 10%. Other more rare types are medullar, tubular, papillary, and mucinous breast cancers (Berg, Hutter 1995). In clinical use, breast cancers are classified into different stages based on the tumor size (T), involvement of the disease in lymph nodes (N), and distant metastasis (M) (Sainsbury et al. 2000). The TNM staging strongly determines the prognosis and is used in the assessment of treatment. Another clinically important feature of breast tumors is differentiation, which is categorized into three grades based on tubule formation, nuclear pleomorphism, and mitotic count, with grade I tumors being well, grade II tumors moderately, and grade III tumors poorly differentiated (Elston, Ellis 1991).

A major molecular classifier of breast cancer is the expression of estrogen and progesterone hormone receptors, ER and PgR, respectively, in the tumor. The expression of these two receptors is strongly correlated with each other and up to 80% of breast cancers are ER positive. The risk factors and biological background for ER negative breast cancer are distinct from ER positive (Duffy 2005). Another molecular classifier of breast cancer is the overexpression of the oncogenic HER2 protein, often through somatic amplification of a genomic region on chromosome 17q containing the *ERBB2* gene, which is a major driver event in 10-34% of breast cancers (Ross et al. 2003).

The proliferation rate of tumor cells can be evaluated by assessing the expression levels of proliferation markers, such as Ki67, which is present in proliferating tissues, but absent in quiescent cells. It is also an important prognostic factor (de Azambuja et al. 2007). The somatic *TP53* mutation is a major driver event in approximately 35% of breast cancer (Cancer Genome Atlas Network 2012). The mutated p53 protein accumulates in cancer cells and can be assessed with immunohistochemical analyses of the protein levels (Borresen-Dale 2003).

In addition to conventional protein markers, gene expression signatures based on microarray data have been applied to divide breast cancers into different groups of so-called intrinsic subtypes, namely luminal A, luminal B, HER2+, normal-like, and basal (Perou et al. 2000, Sorlie et al. 2001). These subtypes are partly correlated with the conventional markers, with luminal subtypes being ER positive, and further separated into luminal A and B with the expression of proliferation-related genes being more frequently overexpressed in the latter. HER2+ tumors are typically ER negative and HER2 positive. Basal cancers are often negative for ER and HER2 and express basal cytokeratines and EGFR (epidermal growth factor receptor). The normal-like subtype resembles normal breast tissue and might, at least partly, be explained by contamination of the tumor sample by normal tissue. Recently even more specific subtypes have been characterized through gene expression profiling combined with copy number variation, classifying breast cancers into 10 categories with survival patterns specific to each group (Curtis et al. 2012).

2.2.2 Prognosis and treatment

Although the incidence of breast cancer has been increasing, the mortality rate of the disease has been decreasing since early 1990s (DeSantis et al. 2011). Early diagnosis improves the outcome, and screening programs have been successful in identifying cases at an early stage. Development of more efficient treatments has also had its effect on the improvement of outcome.

The primary treatment for breast cancer is surgery, either mastectomy, in which the whole breast is removed, or removal of the tumor only with a margin of surrounding healthy tissue. If the regional lymph nodes are affected, they are also removed. Neoadjuvant, or preoperative, chemotherapy or hormonal treatment can be used to reduce the size of the tumor. Following the surgery, radio therapy can be administered on the site of the tumor and regional lymph nodes in patients with high risk of local recurrence of the tumor. Radiotherapy reduces the risk for local or distant recurrence of the disease within 10 years by 15.7% (from 35.0% to 19.3%) and the risk of breast cancer death by 3.8% (Early Breast Cancer Trialists' Collaborative Group (EBCTCG) et al. 2011a).

Systemic adjuvant treatments, such as chemotherapy and endocrine therapy, may be administered in cases with increased risk of recurrence to destroy micrometastatic cancer cells. Adjuvant therapies considerably decrease the risk of recurrence and improve the

survival of the patients, although even severe side effects are not uncommon. Chemotherapy is usually applied as polychemotherapy with a combination of different drugs, for example as a combination of cyclophosphamide, methotrexate and fluorouracil (CMF) or as CEF, in which methotrexate has been replaced with an epirubicine anthracycline. Anthracycline-based treatments have been the most widely used chemotherapy for breast cancer. Taxanes, such as docetaxel, are increasingly added, as they lack cardiotoxicity and improve efficacy compared to anthracycline-alone regimens (Giordano et al. 2012). Poly-chemotherapy reduces the 10 year breast cancer mortality rate by approximately a third (Early Breast Cancer Trialists' Collaborative Group (EBCTCG) et al. 2012).

Other important systemic adjuvant treatments in breast cancer are endocrine therapies, effective in hormone receptor positive cancers, in which estrogen is a major growth factor. Endocrine treatment is used as anti-estrogens (tamoxifen) for both pre- and post-menopausal patients or in post-menopausal patients as inhibitors of aromatase enzyme, which synthesizes estrogen. If tamoxifen is not suitable for a patient, ovarian ablation, by suppressing the ovarian function, can also be applied. Tamoxifen treatment reduces the 15 year breast cancer mortality approximately by 30% and breast cancer recurrency by 15% (Early Breast Cancer Trialists' Collaborative Group (EBCTCG) et al. 2011b). Aromatase inhibitors can, in post-menopausal women, prolong the disease-free survival even more than tamoxifen (Howell et al. 2005, Cuzick et al. 2010).

Finally, HER2 positive tumors can be effectively treated with the systemic HER2 protein-targeting monoclonal antibody trastuzumab, which considerably improves patient survival and decreases the tumor size (Romond et al. 2005, Piccart-Gebhart et al. 2005, Joensuu et al. 2009, Stern 2012). Novel treatments targeting HER2 and other members of the HER family with different mechanisms, for example through other monoclonal antibodies, tyrosine kinase inhibitors, and immunostimulation, are being developed (Arteaga et al. 2011).

2.3 Genetics of breast cancer susceptibility

2.3.1 High-penetrance breast cancer susceptibility genes

2.3.1.1 BRCA1 and *BRCA2*

The most well-known susceptibility genes for hereditary breast cancer are the *BRCA1* (Miki et al. 1994) and *BRCA2* (Wooster et al. 1995). Women with mutations in *BRCA1* have approximately a 57% risk for breast cancer and a 40% risk for ovarian cancer and women with mutations in *BRCA2* have a 49% risk for breast cancer and an 18% risk for ovarian cancer by the age of 70 (Chen, Parmigiani 2007). This risk estimation, however, varies between sample sets and populations, with higher risks seen usually in family based studies already selected for increased risk, possibly through other modifying variants. For rare male breast cancer, men with a *BRCA1* mutation have a risk of 1.2% and men with a *BRCA2* mutation have a risk of 6.8% (Tai et al. 2007). The mutation carriers of *BRCA1* and *BRCA2* are at increased risk of developing pancreatic cancer, but the risk effect is small. *BRCA1* mutation carriers also have slightly increased risk, and *BRCA2* mutation carriers moderately increased risk, of prostate cancer. Some *BRCA2* families may also have elevated risk for melanoma (Levy-Lahad, Friedman 2007).

BRCA1 and *BRCA2* mutations are very rare in the general population, and in breast cancer patients unselected for family history they are found in with a low frequency of 0-7% for *BRCA1* and 1-3% for *BRCA2* (Fackenthal, Olopade 2007). The Breast Cancer Information Core database lists currently 894 *BRCA1* and 824 *BRCA2* pathogenic mutations, of which most are found in single families. Strong founder effects are also seen for example in Finnish, Icelandic, and Ashkenazi Jewish populations (Ferla et al. 2007). A vast majority of the mutations truncate the protein product by causing a premature termination of translation. Small insertions and deletions causing frameshifts, nonsense, and splice site mutations account for the majority of the changes, and particularly in the case of *BRCA1*, large insertions and deletions are common due to the large number of alu-mediated rearrangement hotspots in the introns of the gene. Pathogenic missense mutations are rare (Fackenthal, Olopade 2007). Bi-allelic mutations of *BRCA1* and *BRCA2* are embryonically lethal in mouse models (Gowen et al. 1996, Suzuki et al. 1997), and no bi-allelic *BRCA1* mutation carrier has

been identified in humans. Human bi-allelic mutations in *BRCA2* cause Fanconi anemia subtype FA-D1 (Howlett et al. 2002) (see 2.3.2).

Breast cancers of *BRCA1* and *BRCA2* mutation carriers occur at an early age and are more often bilateral compared to those of familial or unselected non-carriers. Breast tumors of *BRCA1* mutation carriers tend to have histopathological features that are different from non-carrier tumors, but in *BRCA2* mutation carriers, the tumors are more heterogeneous and cannot as clearly be differentiated from non-carrier tumors. *BRCA1*-related breast tumors are more often higher grade (poorly differentiated), and negative for ER and HER2 status. They are often highly proliferative, harbor somatic *TP53* mutations, and over-present the rare medullary histology (Honrado et al. 2006). Somatic mutations in *BRCA1* or *BRCA2* are not common in breast cancer, except in triple negative or basal tumors (Cancer Genome Atlas Network 2012). Epigenetic silencing through hypermethylation of the promoter region of *BRCA1*, however, has been detected in sporadic breast cancer (Esteller et al. 2000).

The BRCA1 and BRCA2 proteins are major regulators of the homologous recombination (HR) DNA double strand break repair machinery; a function they carry out through a complex network of interactions with other proteins (Venkitaraman 2002). This classifies them as caretaker tumor suppressors. BRCA1 can form at least three different functional complexes through its BRCT domain: (i) a complex with abraxas and RAP80, which further interacts with H2AX, RNF8 and MDC1, detecting DNA damage; (ii) a complex with CtiP, which further connects with the NMR complex (NBS1-MRE11-RAD50), also functioning as a sensor for DNA-damage; (iii) a complex with BRIP1 and TOPBP1, which further attach to CHK1 and ATR, functioning in DNA repair during replication (Roy et al. 2011). Furthermore, BRCA1 interacts through its coiled-coil-domain with PALB2, which further interacts with BRCA2, hence linking the two BRCA proteins together. BRCA1 is also involved in estrogen dependent cell proliferation by inhibiting ER-alpha signalling (Fan et al. 1999), and in X-chromosome inactivation (Buller et al. 1999). It participates in cell cycle regulation, is a part of the RNA-polymerase II holoenzyme (Scully et al. 1997), and interacts with the components of the histone-deacetylation complex (Yarden, Brody 1999).

The interactions of BRCA2 are not as versatile as those of BRCA1, with its main functional interaction directly related to HR with RAD51 through the BRC domain. It also interacts with PALB2, which stabilizes BRCA2 and participates in its localization to the site of DNA damage (Roy et al. 2011).

2.3.1.2 Cancer syndromes

Highly increased risk of breast cancer is also characteristic in certain rare dominant autosomal familial cancer syndromes, most notably in Li-Fraumeni syndrome (Malkin et al. 1990), Cowden syndrome (Eng 2003b), and Peutz-Jeghers syndrome (Hemminki et al. 1998), caused by mutations in *TP53*, *PTEN*, and *LKB1* genes, respectively. Li-Fraumeni syndrome is characterized by various cancers with early onset, including sarcomas in bone and soft tissue, leukemias, as well as breast, brain, and adrenal cortex tumors. Of the female Li-Fraumeni patients, 28-56% develop breast cancer by the age of 45 years. Of the families diagnosed with the syndrome, a mutation in *TP53* gene has been identified in 70%. These mutations are typically missense changes, similar to somatic mutations found in *TP53*, with a dominant negative function (Malkin et al. 1990).

Cowden syndrome is a hamartomatous polyposis syndrome with increased risk of malignancies particularly in the thyroid, endometrium, and breast. Life-time risk of breast cancer in Cowden syndrome patients is estimated to be 25-50% (Eng 2003a). Cowden syndrome is caused by inherited mutations in the *PTEN* gene (Nelen et al. 1996, Liaw et al. 1997), which are also associated with Bannayan-Riley-Ruvalcaba syndrome and Proteus syndrome (Eng 2003a). Together these diseases can be classified as *PTEN*-related hamartoma syndromes (PTHS). Recently, activating germline mutations in *PIK3CA* and *AKT*, the proto-oncogenes *PTEN* is regulating, have also been identified in PTHS patients (Orloff et al. 2013). This finding is particularly remarkable as all oncogenenic germline mutations are extremely rare. For more detailed description of the role of *PTEN* in tumor suppression see 2.6.

Another hamartomatous polyposis syndrome with increased risk of breast cancer is Peutz-Jeghers syndrome (PJS), characterized by hamartomatous polyps in the gastrointestinal tract and melanin pigmentation of the lips. PJS patients have increased risk of cancer in the gastrointestinal track, pancreas, breast, and gynaecological sites. The risk of developing breast cancer by the age of 70 is 50% (Hearle et al. 2006). PJS is caused by rare mutations in the *LKB1/STK11* gene (Hemminki et al. 1998).

Finally, mutations in the *CDH1* gene, coding for the E-cadherin protein, cause hereditary diffuse gastric cancer and lobular breast cancer (Schrader et al. 2008). The lifetime risk of *CDH1* mutation carriers to develop lobular breast cancer is between 39-52%.

2.3.2 Moderate-penetrance breast cancer susceptibility genes

Ataxia telengiectasia (AT) is a recessive disease caused by bi-allelelic mutations in the *ATM* gene (Savitsky et al. 1995). The disease is characterized by neuronal degeneration, immunodeficiency, sensitivity to radiation, and strongly increased tumor susceptibility. The cancers of AT patients arise mainly in tissues of lymphoid origin. The ATM protein is a serine/threonine kinase, which participates in the repair of DNA double strand break signalling and interacts with many tumor suppressors such as p53, BRCA1, and BRCA2 (Derheimer, Kastan 2010). Heterozygous mutations in the *ATM* gene increase the risk of breast cancer by approximately two-fold, although higher risk estimates have also been proposed (Ahmed, Rahman 2006). The low frequency of mutations has made exact risk estimation challenging.

Another recessive syndrome genetically related to breast cancer is Fanconi anemia (FA), a rare disorder in which patients suffer from various developmental abnormalities, bone marrow failure, sensitivity to radiation, genomic instability, and congenital defects, and have highly increased risk of various cancers (Auerbach 2009). Fanconi anemia consists of different subtypes originating in bi-allelic mutations in several DNA-repair genes including many which functionally interact with *BRCA1* and *BRCA2*. At least 15 different genes (*FANCA*, *FANCB*, *FANCC*, *FANCD1/BRCA2*, *FANCD2*, *FANCE*, *FANCF*, *FANCG*, *FANCI*, *FANCJ/BRIP1*, *FANCL*, *FANCM*, *FANCN/PALB2*, *FANCO/RAD51C*, and *FANCP/SLX4*) are known to cause distinct FA subtypes, when bi-allelically mutated (de Winter, Joenje 2009, Soulier 2011). Of the FA genes, four, *FANCD1/BRCA2, FANCJ/BRIP1 FANCN/PALB2*, and *FANCO/RAD51C* are also associated with breast cancer susceptibility, when only one of the alleles is mutated. *BRCA2* and *PALB2* function in close interaction, and hence the similar FA phenotype is not surprising. *PALB2* is a moderate-penetrance breast cancer susceptibility gene with the estimated increased risk of breast cancer varying from 2.3-fold (Rahman et al. 2007) to up 6-fold (Erkko et al. 2008), with the true risk likely being somewhere between the results of the two studies.

Monoallelic mutations in *FANCJ,* also known as *BACH1*, or *BRIP1,* are associated with two-fold increased risk of breast cancer (Seal et al. 2006). Bi-allelic mutations in a RAD51 paralog gene *RAD51C* have been identified to account for Fanconi anemia-like disorder (Vaz et al. 2010). The monoallelic mutations in *RAD51C* were also proposed to make it a breast cancer and ovarian susceptibility gene (Meindl et al. 2010), but later we (Pelttari et al. 2011)

and others (Loveday et al. 2012) have shown it to be mainly an ovarian cancer risk gene, with breast cancer only occurring in families also affected with ovarian cancer, a far more rare malignancy than breast cancer. Another *RAD51* paralog *RAD51D* is also an ovarian cancer risk gene (Loveday et al. 2011).

CHEK2 (*Cell Cycle Checkpoint Kinase 2*) is involved in the DNA double strand break response by controlling the cell cycle at several checkpoints, and through interactions with proteins of apoptosis and DNA repair machinery (Bartek, Lukas 2003). A heterozygous truncating mutation 1100delC in the *CHEK2* gene has been identified as a breast cancer susceptibility mutation in many countries, and it is also present in the general population with the highest genotype frequencies seen in the Dutch with 1.3-1.6% (Meijers-Heijboer et al. 2002) and in Finns with 1.4% (Vahteristo et al. 2002). The mutation approximately doubles the risk of breast cancer, but may confer even higher risk in women with a family background of the disease (Nevanlinna, Bartek 2006). Homozygous individuals have also been identified and may have a highly increased risk for breast cancer (Adank et al. 2011). Other truncating variants in the *CHEK2* gene have also been identified with similar effects on breast cancer predisposition (Cybulski et al. 2011). A missense variant, I157T, common in northern and Eastern Europe, with a genotype frequency of 5.3% in Finland, also associates with breast cancer risk, but with lower penetrance than the truncating variants, increasing the risk for breast cancer approximately 1.5 fold (Kilpivaara et al. 2004, Nevanlinna, Bartek 2006).

The number of moderate, but also high-penetrance, variants is likely to grow substantially in the near future as targeted deep sequencing, whole exome sequencing, and eventually whole genome sequencing projects begin to reveal novel rare variants in cancer families. Indeed, targeted sequencing approaches have identified mutations in known susceptibility genes (Walsh et al. 2010), and recently exome sequencing has suggested *XRCC2* to be a breast cancer susceptibility gene (Park et al. 2012) and proposed the same for the *FANCC* and *BLM* genes (Thompson et al. 2012). These findings, however, require rigorous validation in independent patient cohorts, a process often neglected in traditional candidate gene studies as well, and, according to a more recent study, the *XRCC2* variants do not seem to play a major role in familial breast cancer predisposition (Hilbers et al. 2012). The risk estimations and related cancer syndromes of the high and moderate-penetrance breast cancer predisposing genes are summarized in Table 1.

Table 1. High and moderate-penetrance breast cancer susceptibility genes with cancer syndromes they relate to and estimated risk ratios (RR) (Mavaddat et al. 2010).

Gene	Locus	Related syndrome	RR
BRCA1	17q21		5-45
BRCA2	13q12.3	Fanconi anemia (D1)	9-21
TP53	17p13.1	Li-Fraumeni	2-10
PTEN	10q23.1	Cowden	2-10
LKB1/STK11	19p13.3	Peutz-Jeghers	2-10
CDH1	16q22.1		2-10
ATM	11q22.3	Ataxia telengiectasia	2-3
BRIP1	17q22	Fanconi anemia (J)	2-3
PALB2	16p12.1	Fanconi anemia (N)	2-4
CHEK2	22q12.1		2-3

2.3.3 Low-penetrance breast cancer susceptibility genes

A low increase in breast cancer risk has been associated with numerous variants, identified mostly through GWAS and also on a smaller scale, through a candidate gene approach. These variants are typically common in the population and contribute to less than two-fold increased risk of the disease (Fletcher, Houlston 2010). Effects this small require large case-control sample sets and most of the successful studies have been carried out by international consortia, with up to tens of thousands subjects for multi-stage validation. Indeed, many reported associations in single studies have later proven to be false findings when studied in larger independent data sets. The GWAS studies depend on a tagging approach in which SNPs are in linkage disequilibrium (LD) with each other and therefore the risk variant identified cannot be expected to be the actual causative variant. The identification of the causative variant requires fine mapping of the risk locus region and further functional studies. Some of the risk variants, furthermore, localize far away from any known gene in so-called gene deserts, which makes the causative gene hard to identify. Most of the common risk variants are likely regulatory variants affecting the expression levels of their target genes. As these effects are likely to be modest also at the molecular level, and as the high frequency in population indicates no major evolutionary disadvantage, functional studies can also be challenging.

The first low-penetrance risk variants identified for breast cancer susceptibility with strong validation in large independent data sets were two common missense variants from a candidate SNP study: D302H (rs1045485) in the *Casp8* gene with a slight reduction of the risk, and L10P (rs1982073) in the *TGFB1* gene with a slight increase of risk (Cox et al. 2007). Shortly after, the first breast cancer GWAS reported five susceptibility loci, in 5q11.2 near the *MAP3K1* gene, 8q24.2 in an intergenic region upstream of the *MYC* gene, 10q26.1 in the intron two of the *FGFR2* gene, 11p15.5 near the *LSP1* gene, and 16q12.1 close to the *TOX3*/*TNRC9* gene (Easton et al. 2007). To date, variants in altogether 28 loci have been found in genome-wide association studies to be associated with breast cancer risk with high probability (National Cancer Institute 2012). Many associations are stronger in, or even specific to, ER positive or ER negative disease, and some associations are only detected in Asian or European populations. Some common susceptibility variants are also associated with breast tumor subtypes defined by ER, PgR, HER2, CK5/6, and EGFR status (Broeks et al. 2011). No interactions between the genetic variants and the non-genetic risk factors have so far been confirmed, possibly due to the need for a considerably larger sample size to statistically power such analyses (Campa et al. 2011, Milne et al. 2010). The common variation, furthermore, modifies the breast cancer risk of *BRCA1* and *BRCA2* mutation carriers, with different risk variants involved between the mutation carriers of the two genes (Milne, Antoniou 2011).

2.4 Genetics of breast cancer survival

Population-based registry studies from Sweden have shown that a heritable component exists not only for breast cancer risk but also for the prognosis of the disease (Hartman et al. 2007, Lindstrom et al. 2007, Hemminki et al. 2008). The outcome of a breast cancer patient significantly predicts the outcome of breast cancer of her daughters and sisters with a hazard ratio (HR) of 1.6 (95% CI, 1.1-2.3) for daughters and 1.8 (95% CI, 1.0-3.4) for sisters (Hartman et al. 2007). A significantly worse survival rate is also seen in children of parents with poor survival of colorectal, lung, and prostate cancer (Lindstrom et al. 2007). Further evidence for inherited predisposition to cancer outcome has been produced through mouse studies, with the observation that different strains of mice also have different features of mammary tumor progression (Lifsted et al. 1998). The molecular mechanisms behind the familial component of breast cancer survival are still largely unknown. In addition to hereditary variation, giving the tumor a more malignant phenotype at some point during its

evolution and hence affecting the patient survival, the prognostic effect can also be caused by altered response to treatment through, for instance, differences in drug metabolism (Wang et al. 2011).

The effects of *BRCA1* and *BRCA2* mutations on breast cancer prognosis have been under investigation in various studies but the results have been inconclusive. Some studies have found no difference between the survival of *BRCA1* and *BRCA2* mutation carriers compared to non-carriers (Bonadona et al. 2007, Brekelmans et al. 2007, Rennert et al. 2007) but others have found *BRCA1* mutations to be associated with worse survival (Robson et al. 2004, Moller et al. 2007, Lee et al. 2010). The number of *BRCA2* mutation carriers, however, has been very limited in these studies, affecting the power to reliably detect any possible survival effect (Lee et al. 2010, Bordeleau et al. 2010), but a recent study has, however, found associations between these mutations and worse prognosis (Goodwin et al. 2012). In ovarian cancer, both *BRCA1* and *BRCA2* mutation carriers have, interestingly, a better prognosis than the non-carriers, possibly through better response to chemotherapy by DNA damaging agents (Bolton et al. 2012).

The roles other major breast cancer predisposing genes might play in patient survival remain largely unknown. The low frequency of mutations makes survival analyses challenging as the number of events required for survival analyses is often insufficient. The *CHEK2* 1100delT mutation, with a relatively high allele frequency in many populations, has, however, been associated with a 1.4-fold increased risk of early death, 1.6-fold increased risk of breast cancer death, and 3.5-fold increased risk of second breast cancer in ER positive breast cancer patients (Weischer et al. 2012). The low-penetrance risk variants do not seem to be behind breast cancer survival predisposition either. Of the 11 known risk SNPs and 62 other candidate or GWAS related SNPs, only one, rs3803662 at the 16q12.1 locus close to the *TOX3*/*TNRC9* gene, was found to be associated with breast cancer survival in a recent large consortium study, with a HR of 1.21 for overall and 1.29 for breast cancer specific survival for the patients with the rare homozygous genotype (Fasching et al. 2012).

Only a few genome wide association studies have so far been carried out for breast cancer survival. In two studies of breast cancer patients with European ethnicity, no association was detected in the first study (Azzato et al. 2010a), but the second study suggested an association between improved survival and rs4778137 in the *OCA2* gene (Azzato et al. 2010b). A recent Chinese study identified two SNPs associated with breast cancer survival: rs3784099 on

chromosome 14 in intron 7 of the *RAD51L1*/*RAD51B* gene and rs9934948 on chromosome 16 (Shu et al. 2012). A low-penetrance risk SNP, rs999737, not in LD with the survival SNP, has also been identified in *RAD51L1* (Thomas et al. 2009). Another recent study from Japan identified an association between rs10509373 in the *C10orf11* gene on chromosome 10q22 and breast cancer recurrence-free survival in patients treated with tamoxifen (Kiyotani et al. 2012), although the sample set in this study was rather limited in size.

A great number of studies have been published reporting single genetic associations with breast cancer survival, most of which have not been validated in independent datasets. Many studies have been focusing on single candidate genes or variants and some have focused on a larger number of genes, for example within a shared pathway. One example of a suggested treatment response effect is the association of *CYP2D6* variants and breast cancer prognosis through effects on tamoxifen metabolism and treatment response of ER positive tamoxifen-treated patients (Hoskins et al. 2009). Another example is the effect of a missense variant P187S in the *NQO1* gene on the response to epirubicin chemotherapy (Fagerholm et al. 2008).

2.5 *PALB2*

The *PALB2* gene on chromosome 16p12.2 is a major moderate-penetrance breast cancer susceptibility gene (Rahman et al. 2007) in which mutations are also associated with familial pancreatic cancer (Jones et al. 2009). Unlike *BRCA2* mutations, the *PALB2* mutations do not seem to play a role in prostate (Tischkowitz et al. 2008, Pakkanen et al. 2009) or ovarian cancer predisposition (Tischkowitz, Xia 2010), but they have been associated with increased risk of male breast cancer (Casadei et al. 2011). Bi-allelic mutations in the *PALB2* gene cause Fanconi anemia subtype N, as described earlier in 2.3.2 (Xia et al. 2007, Reid et al. 2007), with resemblance to the clinical features of Fanconi anemia subtype D1 caused by the bi-allelic *BRCA2* mutations with high risk of embryonic tumors (Tischkowitz, Xia 2010).

Truncating *PALB2* mutations have been found in breast cancer families in British (Rahman et al. 2007), Finnish (Erkko et al. 2007), French Canadian (Foulkes et al. 2007), Ashkenazi Jewish (Tischkowitz et al. 2007), Spanish (Garcia et al. 2008), Chinese (Cao et al. 2008), South African (Sluiter et al. 2009), Italian (Papi et al. 2010, Balia et al. 2010), Polish (Dansonka-Mieszkowska et al. 2010), Australian (Southey et al. 2010, Wong et al. 2011, Teo et al. 2013a, Teo et al. 2013b), American (Casadei et al. 2011), German (Hellebrand et al. 2011, Bogdanova et al. 2011, Pern et al. 2012), Russian (Bogdanova et al. 2011), and African

American (Zheng et al. 2012) populations. The confirmed pathogenic mutations have so far been protein truncating. The vast majority of the identified mutations have only been identified in single families, but recurrent mutations are also known. The most frequent founder mutation is the Finnish mutation 1592delT, causing a frameshift at leusine residue 531 and a premature stop codon (Erkko et al. 2007). The mutation was originally found to be present in 2.7% of familial breast cancer patients (n=113), 0.9% of unselected patients (n=1918), and in 0.2% of population controls (n=2501). The risk effect of *PALB2* mutations varies depending on the study, from a 2.3-fold increase in the original study identifying *PALB2* as a breast cancer susceptibility gene (Rahman et al. 2007), up to 6-fold risk when using segregation analysis with the Finnish founder mutation (Erkko et al. 2008), an effect comparable to *BRCA2* mutations. Both estimations are based on a very limited set of mutation carriers, making the results unspecific, and particularly the result of the segregation analysis seems an overestimation, biased by its concentration only on a small number of high risk families. Large international collaborative studies are required to reliably estimate the *PALB2* risk in breast cancer and indeed such projects are on-going by the PALB2 Interest Group.

The PALB2 protein (Figure 1) consists of 1,186-amino acids and has a coiled-coil motif at the N-terminal region, with which it interacts with BRCA1, and WD repeats at the C-terminal region forming a β-propeller structure, which interacts with BRCA2 (Xia et al. 2006, Tischkowitz, Xia 2010). The third protein interacting with PALB2 is MRG15 coded by the *MORF4L1* gene, having a binding motif with amino acids 611-764 on PALB2 (Sy et al. 2009a). No mutations in the MORF4L1 gene have, however, been found in breast cancer families (Rio Frio et al. 2010). The main function of the PALB2 protein is to stabilize BRCA2 and to participate in its localization to the site of DNA damage, with approximately 50% of BRCA2 bound to PALB2 (Xia et al. 2006). PALB2 also physically links BRCA1 and BRCA2 together, demonstrating that these proteins work together in HR, joined also by RAD51 (Sy et al. 2009b, Dray et al. 2010). The *Palb2*$^{(+/-)}$ mice do not have a particular phenotype and remained tumor free for the follow-up time of 8 months. *Palb2*$^{(-/-)}$ mice, however, died during embryogenesis with multiple developmental abnormalities, resembling the *Brca1* and *Brca2* knock-out mice (Rantakari et al. 2010). The loss of p53 in Palb2 knock-out mice lessened the phenotype, but did not prevent the embryonic lethality (Bouwman et al. 2011).

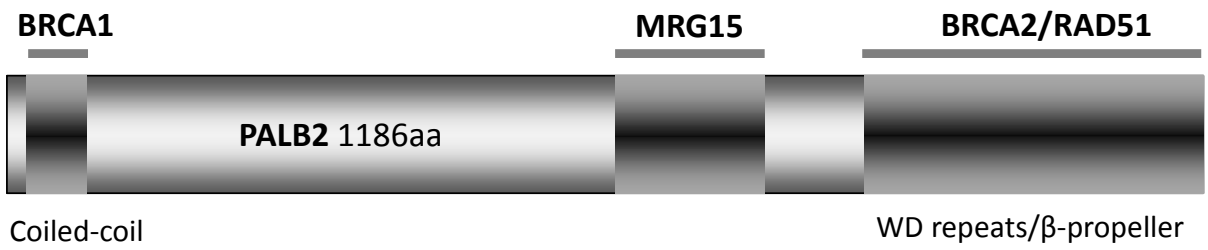

Figure 1. A diagram of the structure of the PALB2 protein with known functional domains and regions binding with interacting proteins. Adapted from (Tischkowitz, Xia 2010).

How *PALB2* mutations affect carcinogenesis has remained uncertain. Loss of heterozygosity (LOH) of the wild type *PALB2* allele has been identified with inconclusive results in different studies, with earlier studies reporting no LOH (Erkko et al. 2007, Tischkowitz et al. 2007) (with five and four tumors analyzed), but later studies have reported it in all tumors analyzed (Garcia et al. 2008, Casadei et al. 2011)(with one and seven tumors analyzed). The dominant negative effect of the truncating mutations with the proteins' HR functions does not seem to be the mechanism, although other dominantly negative effects cannot be excluded (Tischkowitz, Xia 2010). Inactivation of the wild type *PALB2* allele through somatic mutation has been identified in a *PALB2*-related pancreatic cancer case (Jones et al. 2009) and somatic bi-allelic *PALB2* mutation has been reported in a lobular breast cancer case (Shah et al. 2009), although somatic *PALB2* mutations do not seem to be common in breast cancer (Cancer Genome Atlas Network 2012). So far the breast tumors of germline *PALB2* mutation carriers have not been analyzed for somatic *PALB2* mutations, but the second hit through mutation or epigenetic silencing of the wild type allele could be a plausible mechanism. Indeed, hypermethylation has been detected in the promoter region of *PALB2* in sporadic and familial breast cancer and in ovarian cancer, affecting the *PALB2* expression levels (Potapova et al. 2008).

The characteristics of the breast tumors of *PALB2* carriers had prior to this work been analysed only in small number of tumors (Erkko et al. 2007, Foulkes et al. 2007, Tischkowitz et al. 2007, Garcia et al. 2008, Cao et al. 2008). The overall trend towards triple negative tumors, however, has been evident. The effects of *PALB2* mutations on survival of the patients had not been investigated prior to this study.

2.6 *PTEN*

The PTEN protein, a dual specificity phosphatase capable of dephosphorylating both lipids and proteins, is a gatekeeper tumor suppressor, and a key regulator of the activation of the PI3K/AKT oncogenic pathway (Cantley, Neel 1999). The main mechanism of activation of this pathway is the conversion of phosphatidylinositol 4,5-bisphosphate (PIP_2) into phosphatidylinositol 3,4,5-trisphosphate (PIP_3), catalysed by phosphatidylinositol-3-kinase (PI3K). The binding of PIP_3 to AKT allows phosphoinositide dependent kinase 1 (PDK1) to activate AKT through phosphorylation. Actived AKT can then further activate its various targets, related, for example, to cell growth and proliferation (Salmena et al. 2008, Song et al. 2012). In normal conditions the amount of PIP_3 is regulated by PTEN, which dephophorylates it back to PIP_2 and hence limits the activation of AKT (Vivanco, Sawyers 2002). Inactivating mutations or chromosomal loss of the *PTEN* gene, or activating mutations in genes coding PI3K components, most commonly *PIK3CA* for subunit p110α and *PIK3CB* for subunit p85α, or in *AKT1*, cause the pathway to be constantly activated and contribute to the development of cancer (Miller et al. 2011). The alterations of the PI3K pathway seem to be involved in the majority (59%) of sporadic breast tumors (Jones et al. 2013). PTEN also has independent functions outside PI3K/AKT regulation, most notably the negative regulatory functions of the MAPK pathway (Gu et al. 1998). It can be localized in the nucleus through different mechanisms, where it regulates chromosomal stability, acetylates p53, and participates in the DNA-damage response and in the induction of apoptosis (Salmena et al. 2008, Yin, Shen 2008, Planchon et al. 2008, Song et al. 2012).

The *PTEN* gene is located on chromosome 10q23 and it consists of nine exons which code for a 403 amino acid residue protein (Li et al. 1997, Steck et al. 1997). The promoter of *PTEN* is located 5' region of the gene between the nucleotides -1344 and -747 from the translation start site (Figure 2), containing binding sites to transcription factors such as p53, Sp1, and Egr-1 (Sheng et al. 2002, Zhou et al. 2003, Stambolic et al. 2001). Germline mutations in the promoter region of *PTEN* resulting in a decrease of the amount and loss of function of the PTEN protein have been shown to cause Cowden syndrome in a similar manner to inactivating protein coding *PTEN* mutations (Zhou et al. 2003), (see 2.3.1.2). A cis-acting E-box element at position -2181 to -2176 from translation start site of the *PTEN* gene, mediating the transcriptional activity, is often deleted in PHTS patients (Pezzolesi et al. 2007). The PTEN protein has two major domains, the N-terminal phosphatase domain and the C-terminal

domain, which increases protein stability and functions in protein-protein interactions (Lee et al. 1999, Georgescu et al. 2000). The expression level of PTEN is also regulated post-transcriptionally through miRNA binding, which is affected by the expression of a *PTENP1* pseudogene, which, through high level of homology at the 3'UTR region with *PTEN*, acts as a decoy target for miRNAs binding the transcripts (Poliseno et al. 2010).

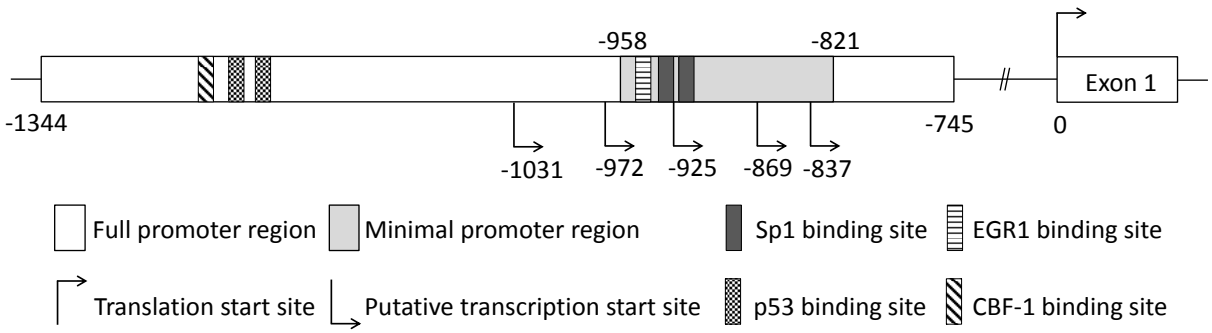

Figure 2. A diagram of the promoter region of the *PTEN* gene with known transcription factor binding sites and putative transcription start sites. The full promoter region is located from nucleotide -745 to -1344 and the minimal promoter region from nucleotide -821to -958 from the translation start site. Adapted from (Teresi et al. 2007).

Somatic mutations in the components of the PI3K pathway are among the most frequent genetic alterations in cancer (Shaw, Cantley 2006). In breast cancer, activating mutations in the *PIK3CA* gene occur in approximately 25% of the cases (Bachman et al. 2004, Perez-Tenorio et al. 2007). The expression of PTEN is lost or reduced in 27-50% of breast cancer (Depowski et al. 2001, Bose et al. 2002, Lee et al. 2004, Torres et al. 2001, Tsutsui et al. 2005, Perez-Tenorio et al. 2007) through mutations or epigenetic mechanisms (Perren et al. 1999, Khan et al. 2004, Bose et al. 2002). PTEN-related tumor suppression is a dosage-dependent mechanism in which even a slight decrease of the amount of the protein may affect the tumor formation (Carracedo et al. 2011, Berger et al. 2011). In mice a decrease of the amount of the PTEN protein to 80% of the normal level increases the susceptibility to cancer, and affected the biology and expression profiles of cancer cell proliferation-related genes in mammary tissue (Alimonti et al. 2010). The elevated expression of PTEN in mice, however, introduced tumor resistance through a metabolic state with tumor suppressive effects (Garcia-Cao et al. 2012). The reduced amounts of expressed PTEN protein in breast tumors have been associated with worse prognosis and aggressive characteristics of the disease (Depowski et al. 2001, Bose et al. 2002, Tsutsui et al. 2005). Similar effects are also seen in tumors with aberrant PI3K/akt pathway (Perez-Tenorio et al. 2007, Lopez-Knowles et al. 2010). Breast

tumors with aberrant PTEN expression present a gene expression signature which is associated with poor prognosis also in prostate and bladder carcinoma (Saal et al. 2007). Loss of PTEN expression is also associated with basal-like breast cancer and inactivation of *Pten* is a driver event for the formation of basal-like mammary tumors in mice (Saal et al. 2008). Particularly in BRCA1 deficient breast tumors, PTEN expression is often lost through gross mutations, such as inversions, deletions, and intragenic chromosome breaks (Saal et al. 2008). Germline mutations in the *PTEN* gene have not been found outside PTHS families (Carroll et al. 1999, Guenard et al. 2007) and common variants of the gene have not been associated with breast cancer risk (Haiman et al. 2006, Guenard et al. 2007).

2.7 eIF4E

One of the major alterations in cancer is aberrant translation of mRNAs (Pandolfi 2004). This is one of the mechanisms which oncoproteins can exploit to increase their levels in tumor cells. A key regulator of translation is the eIF4F complex, consisting of three subunits: eIF4E, eIF4G, and eIF4A. The subunits have specific functions in the complex: eIF4E binds to mRNA 5' cap structures; eIF4A uses its RNA helicase properties to unwind the secondary structures of mRNA and make it accessible to the 43S ribosomal subunits; and eIF4G as a scaffolding protein binds the subunits together (Gingras et al. 1999).

The eIF4F complex regulates translation initiation particularly for transcripts which have long GC-rich 5'UTR structures, including many oncogenic transcripts, for example VEGF, Cyclin D1, and MMP-9. The complex also plays a more general role in translational regulation of transcripts with complex 5' structures (De Benedetti, Graff 2004). A limiting factor to eIF4F formation is the availability of free eIF4E, which is normally bound by 4EBP molecules, restricting the complex formation. The 4EBPs can be phosphorylated by mTOR, a downstream target of the PIK3/AKT pathway, releasing eIF4E to enhance the translation rate (Gingras et al. 2001). The gene expression of *eIF4E* is regulated on the transcriptional level by the Myc oncogene through binding to the promoter region (Ruggero 2009). The suppression of eIF4E expression decreases the growth rate of tumor cells and induces apoptosis in vitro (Dong et al. 2009). The suppression of the eIF4F complex *in vivo* also delayed breast cancer progression and inhibited the translation of VEGF, MMP-9, and Cyclin D1 (Nasr et al. 2013).

Increased levels of eIF4E have been detected in breast, prostate, bladder, cervical, and lung cancer and in head and neck carcinoma (Mamane et al. 2004). In breast cancer its levels have been associated with aggressive tumor characteristics and with worse outcome of the disease (Byrnes et al. 2006, Coleman et al. 2009, Flowers et al. 2009, Li et al. 1998, Holm et al. 2008, McClusky et al. 2005). The prognostic effects of eIF4E have been suggested to be specific to patients with tumors presenting the luminal B subtype (Pettersson et al. 2011). The expression of eIF4E makes, in a mouse lymphoma model, tumors resistant to doxorubicin and mTOR targeting rapamycin treatment with survival effects similar to tumors with activating *Akt* mutations (Wendel et al. 2004). The abnormal translation regulation can also be targeted as a therapy in cancer treatment, and eIF4F complex components, particularly the rate limiting eIF4E, are among the most promising targets (Malina et al. 2011, Graff et al. 2008).

3 Aims of the Study

1. To study the associations between the *PALB2* 1592delT Finnish founder mutation and breast cancer risk, tumor phenotype and patient survival in Southern Finland with analyses also on the tumor phenotype and survival associations of *BRCA1* and *BRCA2* mutations.

2. To investigate the role of germline *PTEN* promoter variation on breast cancer risk, tumor phenotype and patient survival.

3. To evaluate the associations between eIF4E protein expression and characteristics of breast tumors as well as patient survival and treatment outcome.

4 Materials and Methods

4.1 Subjects

4.1.1 Germline DNA samples

In study I, genotypes of *PALB2* mutation 1592delT, and in study II, three *PTEN* promoter polymorphisms were determined from germline DNA samples, isolated from peripheral blood lymphocytes, to study their associations with breast cancer risk, tumor characteristics, and patient survival in a large set of 1,870 unselected and 542 additional familial breast cancer cases and in 1,079 population controls. The unselected series consisted of two cohorts collected in Helsinki University Central Hospital's Department of Oncology in 1997 to 1998 and in 2000 (Syrjakoski et al. 2000, Kilpivaara et al. 2005) and in the Department of Surgery in 2001-2004 (Fagerholm et al. 2008). The first series with 884 patients covered 79%, and the second with 986 patients, 87%, of all consecutive newly diagnosed breast cancer cases at the time of collection. The additional familial cases were collected at Helsinki University Central Hospital's Department of Clinical Genetics (Vehmanen et al. 1997). The familial cases were further classified as belonging to families with strong familial background, with three or more breast or ovarian cancers among first or second degree relatives, including the proband, and to families with the proband and one first degree relative affected with breast or ovarian cancer (Eerola et al. 2000).

The familial cases were tested negative for *BRCA1* and *BRCA2* mutations, with the strong risk families screened for variation in the coding regions and exon-intron boundaries of the genes using denaturing gradient gel electrophoresis (DGGE) with the large exon 11 of *BRCA1* and exons 10 and 11 in *BRCA2* screened for truncating variation using protein truncation test (PTT) and 135 families further for large insertions and deletions using multiplex ligation-dependent probe amplification (MLPA). The rest of the families were screened for the recurrent Finnish *BRCA1* and *BRCA2* mutations (Vahteristo et al. 2001). In study I, samples from 79 *BRCA1* mutation carriers from 40 families and 104 *BRCA2* mutation carriers from 41 families were included. The population controls consisted of 1,286 women from matched geographical regions obtained through Finnish Red Cross Blood Service.

4.1.2 Tumor arrays

The tissue microarrays (TMA) used in Study III to determine the protein expression of eIF4E were constructed from 1,423 paraffin-embedded breast tumors by selecting the most representative from hematoxylin and eosin-stained tumor blocks by a breast cancer pathologist. A set of four 0.6mm cores from each tumor was transferred to an array block (Tommiska et al. 2005). The TMAs had also been used previously to acquire data on biomarkers used in the analyses included in this thesis.

4.1.3 Clinical information and tumor characteristics

All cancer diagnoses were confirmed through the Finnish cancer registry and hospital records. The pathology reports were used for retrieving the data on breast tumor histology and size, and for the presence of nodal and distant metastasis at the time of diagnosis (Eerola et al. 2005). The tumor histology and grade were re-reviewed by a breast cancer pathologist for 1,423 tumors on the TMAs. The tumors were graded according to the Scarff-Bloom-Richardson grading system, modified by Elston and Ellis (Elston, Ellis 1991). Information on ER and PgR statuses was retrieved from pathology reports (Eerola et al. 2005). Samples with more than 10% of the cells stained for ER or PgR by immunohistochemistry (IHC) were considered positive. The HER2 status was based primarily on chromogenic in situ hybridization (CISH) for the samples on the TMAs with six or more replications considered positive and five or less negative. IHC was used when CISH was not available, with samples having <10% of the cells stained considered negative and >90% stained positive (Tommiska et al. 2008).

The expression of the p53 protein was assessed on TMAs using IHC and samples with more than 20% of the cancer cells stained were considered positive (Tommiska et al. 2005). The proliferation marker Ki67 was used to classify the tumors on the TMAs into four categories: ≥30% of cells stained, strong positive expression (3); 20%-29% of cells stained, intermediate; 5%-19% of cells stained, weak positive; and negative with <5% of the cells stained (Ahlin et al. 2007). Data on the expression of cytokeratines 5/6, 14, and 17 were used in study I, with samples having 0-10% of the cells stained in IHC considered negative and 11%-100% stained considered positive (Eerola et al. 2008). Cyclin D1 expression was used in studies I and III and Cyclin E in study III, with samples having more positively stained cells with IHC than the

mean of the cells stained in all tumors (9.1% for Cyclin D1 and 6.8% for Cyclin E) considered positive (Aaltonen et al. 2008).

The tumors were divided into subtypes according to their ER, PgR, and HER2 status with ER or PgR positive, HER2 negative denoted as "ER/PgR+HER2-", ER or PgR positive, HER2 positive denoted as "ER/PgR+HER2+", ER and PgR negative, HER2 positive denoted as "ER&Pgr-HER2+", and ER, PgR, and HER2 negative denoted as "triple negative". In study I a basal-like subgroup was also derived from triple negatives according to the expression of basal cytokratines 5/6, 14, or 17.

The follow-up information for the vital status of the breast cancer patients was obtained from the Finnish Cancer Registry and from hospital records. The follow-up information on distant metastasis (II) was retrieved from hospital records. The survival was calculated as time between the primary surgery and end of follow-up or breast cancer death within 10 years, and in study II also between the primary surgery and end of follow-up or breast cancer death or distant metastasis (BDDM) within five years from diagnosis. In study I, 2,342 patients with invasive breast cancer were included in the survival analysis with a median follow-up time of 75 months and 284 patients dying of breast cancer within 10 years. In study II, 2,204 patients were included with a median follow-up time of 83 months for 10 year breast cancer-specific death with 298 events and 47 months for BDDM with 352 events. In study III, 1,028 tumors were included in the 10 year breast cancer-specific death analysis with a median follow-up time of 100 months and with 175 events.

4.1.4 Fresh-frozen tumor samples

In study II, 183 fresh frozen breast tumors were used for gene expression microarray analyses. Of the tumors, 151 belong to the patients in the unselected series and 52 to the additional familial cases. The total RNA was extracted from the tumor samples using mirVANA RNA isolation kit (Life Technologies, Carlsbad, CA, USA). The samples were hybridized to Illumina Human HT-12 v3 Expression Bead Chips (Illumina Inc, San Diego, CA, USA). The data acquired in this study was submitted to the Gene Expression Omnibus (GEO) database (Edgar et al. 2002) according to the MIAME protocol (Brazma et al. 2001) (GSE24450).

4.2 DNA analysis methods

In study I, the *PALB2* Finnish founder mutation 1592delT was genotyped in germline DNA samples using AmpliFluor fluorescent genotyping (KBiosciences, Cambridge, UK) or with conformation sensitive gel electrophosresis (CSGE) heteroduplex analysis (Ganguly 2002). In CSGE PCR-amplified DNA fragments are first denaturated for 10 minutes in 95°C and then cooled down to room temperature slowly. This creates heteroduplexes between the DNA strands with mismatches, which have a distinct mobility patterns in a gel compared to homoduplexes. The heteroduplex samples were run in 10% acrylamide, 10% ethylene glycol, and 15% formamide mildly denaturing gels with 2W overnight or 30W for 4 hours and visualized with silver staining.

In study II, the promoter region of the *PTEN* gene was screened for variation in 330 patients using CSGE. The detected variants were further genotyped in the whole sample set using CSGE for two of the variants (-903GA and -975GC) and Sequenom i-PLEX (Sequenom, San Diego, CA, USA) for -1026CA with service provided by FIMM (Institute for Molecular Medicine Finland) Genomics services (www.fimm.fi).

All variants detected with CSGE in studies I and II were verified with direct sequencing. The PCR products were first purified using ExoSAP-IT treatment (Affymetrix, Santa Clara, CA, USA) and then sequenced with ABI BigDyeTerminator 3.1 Cycle sequencing kit (Life Technologies). The sequencing reactions were analyzed either by using the service provided by FIMM Sequencing services with ABI3730xl DNA Analyzer (Life Technologies) or by running them after acetate ethanol precipitation in AB Prism 310 Genetic Analyzer automated sequencer (Life Technologies).

4.3 Immunohistochemical methods

The expression of the eIF4E protein in study III was evaluated using IHC. The TMA slides were deparaffinizated and hybridized with eIF4E rabbit monoclonal antibody (Cell Signalling Technology, #2067, Danvers, MA, USA) diluted 1:400 and stained using an automated Ventana Benchmark immunostaining device (Ventana Medical Systems, Oro Valley, AZ, USA). The IHC staining intensity was scored semi-quantitatively (low, intermediate, and high) two times by two researchers (T.K. and O.C.). All four cores of each tumor sample on TMA were scored individually and in the cases where the result varied the most frequent

scores were used. Unsuccessfully stained samples and samples with no tumor cells were excluded.

4.4 Statistical methods and bioinformatics

4.4.1 Associations with risk and tumor characteristics

The risk analyses and the association analyses between tumor characteristics and genotypes in studies I and II and with protein expression in study III were performed using SPSS 15.0 software (SPSS Inc, Chicago, IL, USA). Pearson's X^2 test or Fisher's exact test, when the number in any of the cells was less than five, was used to calculate the *P*-values. In study III linear-by-linear association X^2 was used for ordinal and binary variables. The associations with *PALB*2, *BRCA1*, and *BRCA2* mutations and the age of onset of breast cancer were assessed with student's t-test. All *P*-values are two sided.

4.4.2 Survival analyses

In studies I and II, the survival analyses were performed using SPSS 15.0. Univariate analyses were calculated with logrank test and visualized with Kaplan-Meier plots. Hazard ratios were calculated with Cox's regression. To test the independence of the variables from the common prognostic factors (tumor size (T), nodal status (N), metastasis at diagnosis (M), ER, PgR, HER2, p53, Ki67, and grade) multivariate models were constructed, with a forward conditional algorithm in study I and a backward conditional in study II. In study II, the three promoter variants were combined in multivariate analysis as a single variable to increase the statistical power.

In study III, the survival analyses were performed with the R environment (www.r-project.org) Survival package (Therneau 2002). The survival times were left-truncated for the time of entry to the study in prevalent cases. Univariate effects of the eIF4E expression were evaluated with Kaplan-Meier analyses with logrank test. The multivariate model was adjusted for tumor size, nodal status, metastasis at diagnosis, ER, PgR, grade, and age of diagnosis. The tumor characteristics (ER, PgR, HER2, Nodal status, grade, p53, Ki67) and treatment (chemotherapy, anthracycline, CMF, and endocrine treatment) were further used to stratify the survival analyses into mutually exclusive subgroups which were tested for heterogeneity with two-sample z-tests on the coefficients (log(HR)) of corresponding univariate Cox models.

4.4.3 Gene expression microarray analyses

In study II, the raw data was imported into the R environment and analyzed using BioConductor packages (Gentleman et al. 2004) with quality control (Du et al. 2008) and normalization by the quantile method (Bolstad et al. 2003). The primary breast tumors of ten *PTEN* promoter variant carriers and 10 non-carriers matched with ER, PgR, HER2, Grade tumor size, p53 and Ki67 were compared for gene expression matrix for the average of probes matching the same Entrez gene IDs and the differential expression was evaluated by moderated t-test, with $P < 0.01$ considered significant (Smyth 2005). The differentially expressed genes were functionally annotated using DAVID functional annotation tools (Huang da et al. 2009) with categories having Fisher's exact *P*-value < 0.05 considered as significant enrichment.

The 160 genes differentially expressed between the tumors of *PTEN* promoter variant carriers and matched non-carriers were used to cluster the set of 183 tumors (GSE24450) into two groups with unsupervised clustering based on the overall expression of the signature genes (Lukes et al. 2009) and analyzed for breast cancer survival or occurrence of distant metastasis within five years from diagnosis with Kaplan-Meier analysis and Log-Rank test. A pre-defined random number selection algorithm was imposed at the beginning of the process to stabilize the results with 100,000 iterations of the K-means algorithm. Unsupervised clustering was further applied to divide three additional independent breast cancer datasets retrieved from GEO database (GSE1456 from Stocholm (Pawitan et al. 2005), GSE2034 from Rotterdam (Wang et al. 2005), and GSE4922 from Uppsala (Ivshina et al. 2006)) into two categories for the validation of the survival effects of the signature genes.

4.5 Ethical aspects

All studies were performed with informed consent from the patients and permissions from the ethics committee of the Helsinki University Central hospital and the Ministry of Social Affairs and Health in Finland.

5 Results

5.1 *PALB2* 1592delT

5.1.1 Breast cancer risk

The *PALB2* 1592delT allele was present in altogether 12 (0.7%) of the 1,706 unselected patients, 19 (2.0%) of the 947 familial cases, and 2 (0.2%) of the 1,079 population controls genotyped *(I, Table 1)*, indicating a trend of increased risk with mutation carriers with increasing family history of breast cancer (Figure 3). The mutation was also found in two unrelated *BRCA2* mutation carriers (with c.8947dupG, p.Asp2983fs and c.7480C>T, p.Arg2494X mutations) of the 104 screened, but not in any of the 79 *BRCA1* mutation carriers tested.

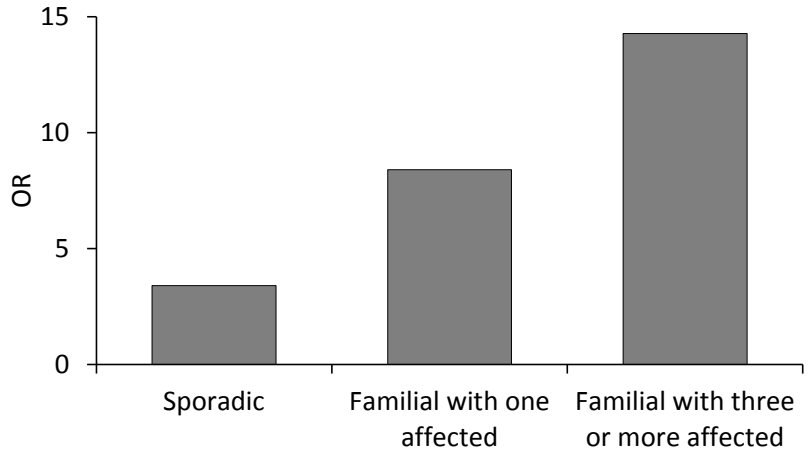

Figure 3. OR of *PALB2* 1592delT in patients with increasing familial burden of breast cancer with familial with three or more affected OR = 14.27 (95% CI 3.09-132.82, $P = 3.69 \times 10^{-5}$), familial with two affected OR = 8.40 (95% CI 1.67-81.34, $P = 0.0028$), and in sporadic OR = 3.40 (95% CI 0.68-32.97, $P = 0.1207$).

The mean age of onset of breast cancer of the carriers of the *PALB2* 1592delT mutation was 53.1 years (33.4–79.9 years), not significantly different from that of familial (54.8. years $P = 0.472$) or sporadic (58.0 years $P = 0.152$) breast cancer patients. In *BRCA1* and *BRCA2* carriers, the age of onset was, however, significantly lower than in non-carriers, at 45.2 years and 47.4 years, respectively ($P < 0.001$). Contralateral breast cancer was present at a similar frequency in *PALB2* mutation carriers and other familial cases, with 3 of 28 (10.7%) *PALB2* carriers having contralateral breast cancer compared to the frequency of 10.9% in the familial cases ($P = 0.99$).

5.1.2 Tumor features

The *PALB2* mutation was associated with numerous tumor properties of aggressive breast cancer. These tumors were more frequently of a higher grade, had higher expression of the Ki67 proliferation marker, and were more often ER and PgR negative (Table 2). All but one tumor of 22 *PALB2* mutation carriers (4.5%) with HER2 data were negative for HER2 overexpression/amplification, but the difference was not statistically significant when compared to familial or sporadic cases. The *PALB2* mutation status also associated strongly with the clinically challenging triple negative phenotype. The expression of cytokeratines alone was associated with the *PALB2* mutation only for cytokeratine 14 when compared to sporadic cases, although the overall trend of cytokeratine expression seemed to be more frequently high in the tumors of *PALB2* mutation carriers. The basal-like subtype separated from the triple negatives by cytokeratine expression was also more frequent in *PALB2*-related tumors compared to the tumors of non-carriers (OR = 8.29, 95% CI 3.26-21.03, $P = 1.388 \times 10^{-7}$). The expression of Cyclin D1 and Cyclin E was more often low in *PALB2* carriers.

To compare the tumor phenotype of *PALB2* mutation carriers to *BRCA1* and *BRCA2* carriers, the analyses were also performed on the mutation carriers of those genes *(I, supplementary Table 1)*. The tumors of *BRCA1* carriers were even more frequently negative for ER (74.6%) and PgR (80.6%) than *PALB2* mutation carriers but had less often lymph node metastasis at diagnosis (28.2%). Similar to the tumors of *PALB2* mutation carriers, the *BRCA1* and *BRCA2* mutation carriers very rarely had HER2 positive tumors, with only one *BRCA1* carrier (2.4%) having a HER2 positive breast cancer. The *BRCA1*-related tumors were also more often triple negative (70.0%) and basal-like (27.5%) compared to the tumors of familial or sporadic patients, but the tumors of *BRCA2* carriers were more frequently of the ER/PgR+HER- subtype (77.3%) (Figure 4 C). With tumor grade and Ki67 expression, *PALB2* tumors seem to lie between *BRCA1* and *BRCA2*-related tumors (Figure 4 A and B).

Table 2. Comparison of tumor characteristics of *PALB2* 1592delT mutation carriers and non-carriers (WT).

Category	*PALB2* 1592delT	%	WT	%	*P*-value	OR	95% CI
T							
1	20	(60.6%)	1375	(61.3%)	0.9325	0.97	0.48-1.96
2, 3 & 4	13	(39.4%)	867	(38.7%)			
N							
negative	15	(45.5%)	1248	(55.9%)	0.2286	0.66	0.33-1.31
positive	18	(54.5%)	983	(44.1%)			
M							
negative	32	(97.0%)	2180	(96.6%)	> 0.9999	1.13	0.15-8.38
positive	1	(3.0%)	77	(3.4%)			
ER							
negative	14	(46.7%)	398	(18.5%)	0.0001	3.86	1.87-7.98
positive	16	(53.3%)	1757	(81.5%)			
PR							
negative	17	(56.7%)	733	(34.0%)	0.0096	2.53	1.22-5.24
positive	13	(43.3%)	1420	(66.0%)			
HER2							
negative	21	(95.5%)	1070	(84.9%)	0.2328	3.73	0.50-27.89
positive	1	(4.5%)	190	(15.1%)			
p53							
negative	18	(78.3%)	976	(79.7%)	0.8676	0.92	0.34-2.50
positive	5	(21.7%)	249	(20.3%)			
grade							
1	3	(9.4%)	574	(26.7%)	0.0018	0.34	0.17-0.69
2	12	(37.5%)	972	(45.2%)	1 and 2 vs 3		
3	17	(53.1%)	604	(28.1%)			
tumor histology							
ca ductale	25	(75.8%)	1526	(67.3%)	0.3041	1.52	0.68-3.38
ca lobulare	3	(9.1%)	481	(21.2%)	Ductal vs other		
ca medullare	0	(0.0%)	28	(1.2%)			
other	5	(1.,2%)	232	(10.2%)			
Type							
ER/PgR+HER2-	9	(40.9%)	900	(73.8%)	$2.217x10^{-10}$	9.88	4.19-23.32
ER/PgR+HER2+	1	(4.5%)	118	(9.7%)	Triple-neg vs other		
ER&PgR-HER2+	0	(0.0%)	69	(5.7%)			
TripleNeg	12	(54.5%)	132	(10.8%)			
Ki67							
0	3	(10.3%)	430	(21.5%)	0.0023	0.32	0.15-0.69
1	7	(24.1%)	814	(40.7%)	0 and 1 vs 2 and 3		
2	8	(27.6%)	373	(18.7%)			
3	11	(37.9%)	382	(19.1%)			
Cyclin E							
High	9	(40.9%)	163	(16.9%)	0.0034	3.40	1.43-8.08
Low	13	(59.1%)	800	(83.1%)			
Cyclin D1							
High	7	(31.8%)	511	(52.8%)	0.0509	0.42	0.17-1.03
Low	15	(68.2%)	456	(47.2%)			

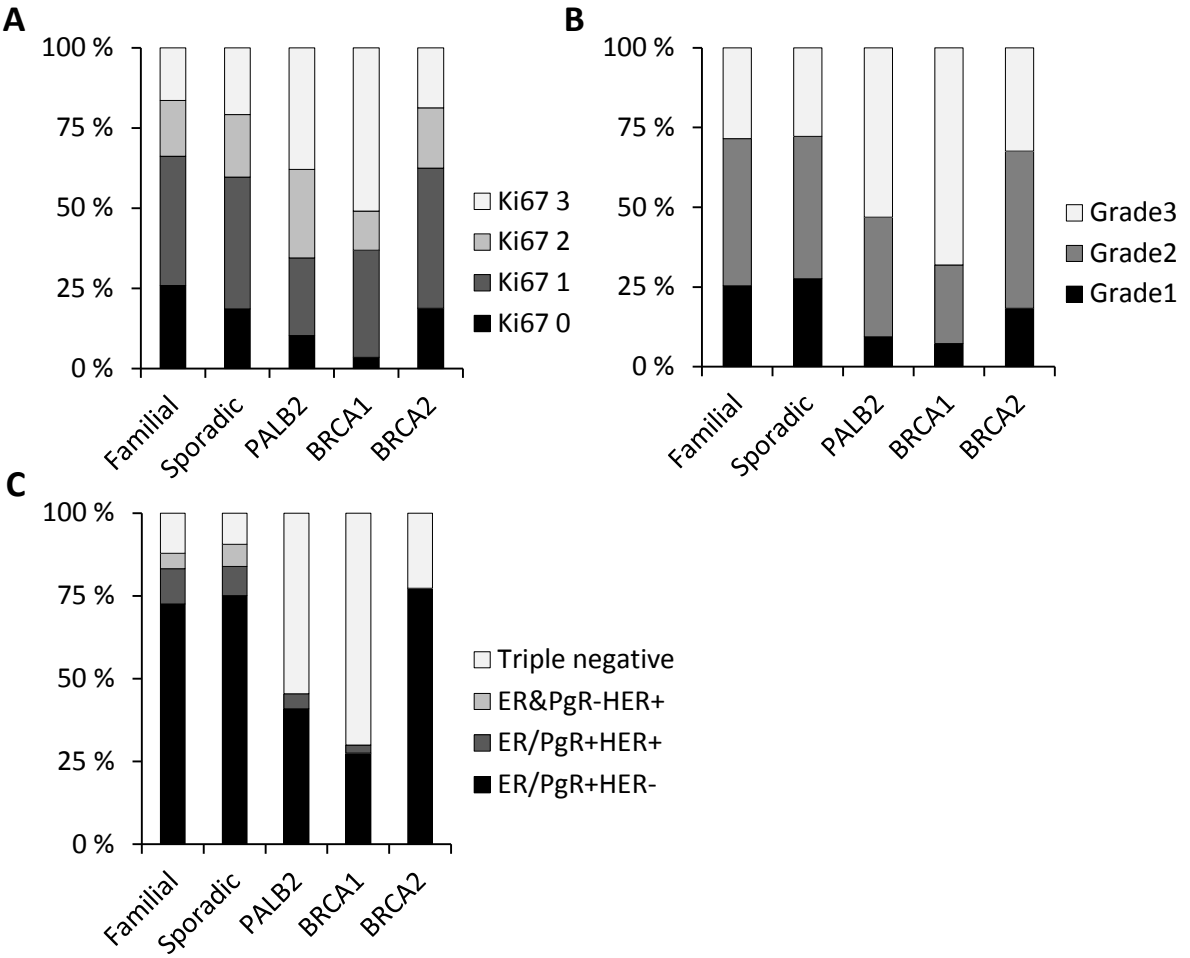

Figure 4. The subtype (A), grade (B), and Ki67 (C) distribution, in the familial, sporadic, *PALB2*, *BRCA1*, and *BRCA2*-related breast cancer.

5.1.3 Survival

The association between survival and the *PALB2* mutation was analyzed together with *BRCA1* and *BRCA2* mutations and compared with familial and sporadic patients. As familial and sporadic cases did not show differences in survival, the groups were combined in the analyses when compared to mutation carriers. The *PALB2* mutation carriers had a survival effect (HR = 1.61, 95% CI 0-71-3.61, *P* = 0.2524) similar to *BRCA1* carriers (HR = 1.67, 95% CI 0.99-2.82, *P* = 0.0546) in the primary analysis, but neither of the effects were significant *(I, Table 3)*. *BRCA2* mutation carriers, however, had a strongly increased risk of death of breast cancer in the long-term 10 year analysis (HR = 2.34, 95% CI 1.50-3.66, *P* = 0.0002), with the survival difference becoming noticeable only after five years of follow-up *(I, Figure 1 A)*.

As the HER negative tumors were a major subgroup among the mutation carriers of all three genes, the survival analyses were stratified, with HER2 status producing a similar survival pattern between *BRCA2* (HR = 3.75, 95% CI 2.18-6.45, $P = 1.781 \times 10^{-6}$) and *PALB2* (HR = 2.94, 95% CI 1.29-6.69, $P = 0.0104$) mutation carriers *(I, Figure 1 B)*. *BRCA1* carriers, however, did not have a survival effect that was different from non-carriers in this subgroup ($P = 0.3592$). The association of the *PALB2* mutation with survival was further analyzed in familial cases only, among which it had a significant association with prognosis (HR = 2.30, 95% CI 1.01-5.24, $P = 0.0466$) *(I, Figure 1 C)*, and also in familial HER2 negative cases where the strongest survival effect for *PALB2* mutation was seen (HR = 4.57, 95% CI 1.96-10.64, $P = 0.0004$) *(I Figure 1 D)*.

In the multivariate analysis adjusted for common prognostic factors *(I, Table 4)*, carrying a *BRCA2* mutation was a strong independent prognostic factor, together with grade, T, N, M, PgR, and HER2, in the final step of the Cox's regression model increasing the risk of dying of breast cancer by two-fold within the 10 years from diagnosis in all cases ($P = 0.0418$) and in HER2 negative cases, together with T, N, M, PgR, and p53, ($P = 0.0459$). Carrying a *PALB2* mutation was an independent prognostic factor in HER2 negative cases, together with T, N, M, PgR, and p53, with 2.5-fold risk ($P = 0.0399$), in familial cases, together with T, N, M, and ER, with 2.4-fold risk ($P = 0.0505$), and with familial HER2 negative cases, together with T, N, M, and ER, with 3.5-fold risk ($P = 0.0082$) of dying of breast cancer within the follow-up time.

5.2 *PTEN* promoter variants

5.2.1 Detection and breast cancer risk

Three variants were detected when the promoter region of the *PTEN* gene was screened using CSGE in a set of 330 breast cancer patients with strong family history of breast or breast and ovarian cancer. Two of the variants, -1026CA (rs34149102) and -903GA (rs1044322), were previously known low-frequency polymorphisms, and one, -975GC, a novel variant, with the number representing the distance from the translation start-site.

In the genotyping of the complete dataset the variants were present with frequencies of 2% for -1026CA, 1% for -975GC, and 3% for -903GA. Two patients had both -1026CA and -903GA and one patient both -903GA and -975GC variants. The promoter variants did not associate with breast cancer risk when compared to population controls in unselected or

familial cases, although a very low increase in risk cannot be excluded (Table 3) (Heikkinen et al., unpublished results).

5.2.2 Tumor features

The -975GC variant was associated with distant metastasis at diagnosis (OR = 4.99, 95% CI 1.69-14.78, *P* = 0.013) and -1026GA with high expression of Ki67 (OR = 2.21, 95% CI 1.15-4.28, *P* = 0.015) when compared to the tumors of non-carriers *(II, Table S1)*. No other significant associations were detected with the promoter variants and tumor characteristics tested (tumor size, nodal status, grade, histology, ER, PgR, HER2, and p53).

Table 3. Frequencies of the three *PTEN* promoter variants and of carriers of any of the variants in breast cancer cases and controls compared to non-carriers (Heikkinen et al., unpublished results).

PTEN -903GA	Total	GG	(%)	GA	(%)	p-value	OR	95% CI
Population controls	1272	1241	(97.6)	31	(2.4)			
Familial, two affected	522	506	(96.9)	16	(3.1)	0.449	1.27	(0.69-2.33)
Familial, three or more affected	429	416	(97.0)	13	(3.0)	0.503	1.25	(0.65-2.41)
All familial breast cancer cases	951	922	(97.0)	29	(3.0)	0.378	1.26	(0.75-2.10)
Unselected breast cancer patients	1709	1662	(97.2)	47	(2.8)	0.596	1.13	(0.72-1.79)
Sporadic breast cancer patients	1286	1253	(97.4)	33	(2.6)	0.835	1.05	(0.64-1.73)

PTEN -975GC	Total	GG	(%)	GC	(%)	p-value	OR	95% CI
Population controls	1272	1258	(98.9)	14	(1.1)			
Familial, two affected	522	516	(98.9)	6	(1.1)	0.929	1.04	(0.40-2.73)
Familial, three or more affected	429	422	(98.4)	7	(1.6)	0.389	1.49	(0.60-3.72)
All familial breast cancer cases	951	938	(98.6)	13	(1.4)	0.571	1.25	(0.58-2.66)
Unselected breast cancer patients	1709	1692	(99.0)	17	(1.0)	0.778	0.90	(0.44-1.84)
Sporadic breast cancer patients	1286	1270	(98.8)	16	(1.2)	0.736	1.13	(0.55-2.33)

PTEN -1026CA	Total	CC	(%)	CA	(%)	p-value	OR	95% CI
Population controls	1260	1244	(98.7)	16	(1.3)			
Familial, two affected	523	515	(98.5)	8	(1.5)	0.665	1.21	(0.51-2.84)
Familial, three or more affected	423	415	(98.1)	8	(1.9)	0.351	1.50	(0.64-3.53)
All familial breast cancer cases	946	930	(98.3)	16	(1.7)	0.413	1.34	(0.67-2.69)
Unselected breast cancer patients	1719	1685	(98.0)	34	(2.0)	0.137	1.57	(0.86-2.85)
Sporadic breast cancer patients	1294	1268	(98.0)	26	(2.0)	0.142	1.59	(0.85-2.99)

PTEN promoter variant	Total	wt	(%)	variant	(%)	p-value	OR	95% CI
Population controls	1252	1191	(95.1)	61	(4.9)			
Familial, two affected	520	491	(94.4)	29	(5.6)	0.538	1.15	(0.73-1.82)
Familial, three or more affected	423	396	(93.6)	27	(6.4)	0.229	1.33	(0.83-2.12)
All familial breast cancer cases	943	887	(94.1)	56	(5.9)	0.271	1.23	(0.85-1.79)
Unselected breast cancer patients	1700	1604	(94.4)	96	(5.6)	0.354	1.17	(0.84-1.62)
Sporadic breast cancer patients	1278	1204	(94.2)	74	(5.8)	0.304	1.20	(0.85-1.70)

5.2.3 Survival

All three *PTEN* promoter variants associated with decreased breast cancer specific survival in the 10 year analyses with P = 0.016 for -903GA, P = 0.002 for -975GC, and P = 0.014 for -1026CA *(II, Figure 1 A)*. The variants -903GA and -975GC also associated with five year breast cancer death or distant metastasis-free survival with P = 0.002 and P = 0.010, respectively *(II, Figure 1 B)*. In Cox's regression analysis, carrying any of the variants approximately doubled the risk of dying of breast cancer within 10 years (HR = 2.17, 95% CI 1.52-3.10, P = 0.00002) or developing metastasis, or dying of breast cancer in five years from diagnosis (HR = 1.97, 95% CI 1.40-2.79, P = 0.00011) *(II, Table 1)*, with the effects visualized in Figure 5 with Logrank *P*-values. In the multivariate analyses, carrying any of the promoter variants was an independent prognostic factor when adjusted by the established prognostic markers in the final step of the Cox's regression, together with T, N, M, grade, and PgR in 10 year breast cancer specific analysis (P = 0.0119) and in five year breast cancer death or distant metastasis-free analysis, together with T, N, grade, and PgR (P = 0.0381) *(II, Table 2)*.

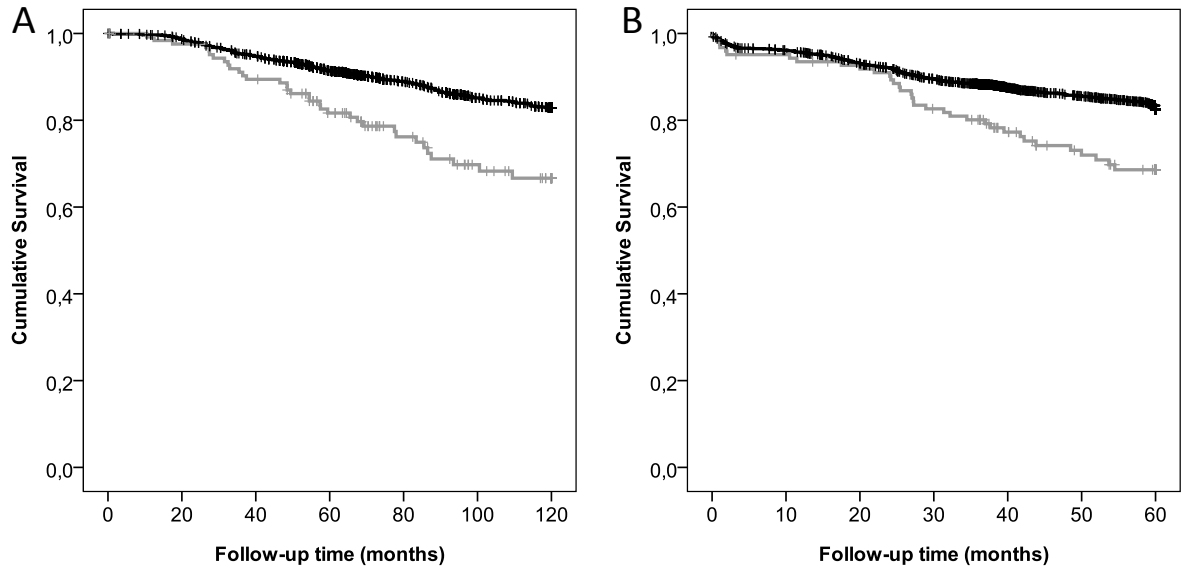

Figure 5. Kaplan-Meier plots of cumulative survival for carrying any of the three *PTEN* promoter variants (grey) compared to wild type (black) for ten year breast cancer death specific survival (A) ($P = 1.088 \times 10^{-5}$) and for five year breast cancer death or distant metastasis free survival (B) ($P = 1.974 \times 10^{-4}$).

5.2.4 Gene expression signature

The microarray analysis of the tumor samples of 10 *PTEN* promoter variant carriers and 10 matched non-carriers revealed a signature of 160 differentially expressed genes *(II, Table S2)*, with $P < 0.01$, although none of them remained significant after *post hoc* correction. In the tumors of the promoter variant carriers, 56 if the genes showed decreased, and 104 increased, expression levels, with changes spanning between 1.81 and -1.85 base 2 log fold (3.5/-3.6 in natural scale). Clustering of the subjects with the expression profiles of the 160 genes co-segregated within branches of hierarchical clustering *(II, Figure 2)*. No segregation was present when the complete expression matrix of 24,660 genes was used in hierarchical clustering. No differences in the mRNA levels of the *PTEN* gene was detected between the tumors of the promoter variant carriers and non-carriers.

The annotation of the differentially expressed genes linked the genes with increased expression in the tumors of the *PTEN* promoter variant carriers to ATP-binding, protein phosphorylation, and protein kinases. In down-regulated genes, DNA-binding and transcription factor proteins were over-represented functional groups *(II, Table S3)*. When compared to 150 genes in the expression signature of somatic *PTEN* deficiency in breast tumors (Saal et al. 2007) only one, *TUBB2C*, was shared with the signature identified here. On the functional level, the signatures, however, shared biological themes such as phosphoprotein and ATP-binding in up-regulated genes and DNA-binding and transcription regulation in down-regulated genes.

The gene expression dataset of 183 breast tumors and three independent publically available datasets were used to evaluate the effect of the gene signature on breast cancer survival. Unsupervised clustering of the signature genes divided the sample sets into two groups with significant association with survival in the Helsinki, Stockholm, and Rotterdam datasets *(II, Figure 3)*. When the clustering was done using the gene expression signature of somatic *PTEN* loss (Saal et al. 2007), a significant survival effect was seen in all four data sets (Helsinki $P = 1.06 \times 10^{-6}$, Stockholm $P = 0.003$, Rotterdam $P = 0.001$, and Uppsala $P = 0.002$). The Saal signature and the *PTEN* promoter variant signature described in this study clustered 93%, 74%, 69%, and 76% of the tumors in corresponding survival groups in the Helsinki, Stockholm, Rotterdam, and Uppsala datasets, respectively.

5.3 eIF4E protein expression

5.3.1 Tumor features

Of the 1,085 successfully stained tumors, 102 showed high, 518 moderate, and 465 low eIF4E IHC staining *(III, Figure 1)*. Increasing eIF4E protein expression associated with higher tumor grade, larger tumor size, ER and PgR negativity, p53 positivity, and higher levels of Ki67, Cyclin E, and Cyclin D1. The breast tumors with high eIF4E IHC were also more often medullary for histological type than tumors with low or intermediate expression (OR = 6.28, 95% CI 2.02-79.58, *P* = 0.0003) *(III, Table 1)*. High eIF4E, furthermore, associated with higher frequency of triple negative (OR = 2.45, 95% CI 1.50-3.99, *P* = 0.0002), ER/PgR+HER2+ (OR = 2.41, 95% CI 1.35-4.29, *P* = 0.0021), or ER&PgR-HER2+ (OR = 3.29, 95% CI 1.65-6.57, *P* = 0.0004) tumor subtype, and were less often of ER/PgR+HER2- subtype. The eIF4E expression did not associate with family history of breast cancer when comparing the tumors of familial and sporadic patients with high and intermediate or low eIF4E (*P* = 0.3030).

5.3.2 Survival

High expression of eIF4E was associated with worse breast cancer death specific survival in the 10 year analysis when compared to low or intermediate eIF4E expressing tumors (HR = 1.99, 95% CI 1.32-3.00, *P* = 0.0008) *(III, Figure 3 A)*. There was no difference in the survival of the patients with tumors with low or intermediate eIF4E expression (HR = 0.97, 95% CI 0.71-1.35, *P* = 0.8779) and therefore the two groups were combined for the analyses. High eIF4E was also an independent prognostic factor in multivariate analysis adjusted for T, N, M, grade, ER, and age of diagnosis (*P* = 0.0400) *(III, Table 2)*.

In subgroup analyses, the dataset was stratified by ER, PgR, HER2, Nodal status, grade, p53, and Ki67, as well as by chemotherapy and endocrine treatment *(III, Table 3)*. From the treatment subgroups, the strongest hazard was seen in the subgroup of patients treated with anthracycline-based chemotherapy (HR = 3.34, 95% CI 1.72-6.48, *P* = 0.0002). In patients not receiving chemotherapy or patients treated with CMF no differential survival effect was observed. The difference of the survival effects in the subgroup of patients treated with anthracycline was also significant in heterogeneity analysis compared to patients not treated with anthracycline (*P* = 0.0358) or who had not received any chemotherapy (*P* = 0.0481). The survival associations were consistent in subgroups of different tumor characteristics, with

high eIF4E being associated with worse survival. The survival effect was strongest in the subgroup of nodal positive patients (HR = 2.68, 95% CI 1.68-4.29, $P = 1.76 \times 10^{-5}$). There was no significant difference in heterogeneity in the tumor characteristic subgroups. High eIF4E expression associated also with survival in metastasized breast cancer (HR = 1.88, 95% CI 1.20-2.94, $P = 0.0060$) in the five year analysis after detection of distant metastasis compared to low or intermediate expression.

6 Discussion

6.1 *PALB2*

The Finnish *PALB2* founder mutation was identified in 2.0% of familial and 0.7% of unselected breast cancer patients and in 0.2% of population controls. The frequencies seen here are in line with previous studies of the mutation in breast cancer patients from Northern Finland (Erkko et al. 2007). The increase in risk of breast cancer caused by *PALB2* mutations remains to be clarified, but it seems to be higher (Erkko et al. 2008, Casadei et al. 2011, Tischkowitz et al. 2012) than the approximately two-fold increase first reported (Rahman et al. 2007). The results here show a trend of increasing frequency of the mutation with increasing family history, consistently with a multiplicative model of hereditary breast cancer predisposition, which might also have affected the high risk estimates reported for *PALB2*-related breast cancer risk in family based segregation analysis (Erkko et al. 2008, Tischkowitz, Xia 2010). In the multiplicative model, a number of yet unknown susceptibility alleles enriched in families may act together with the known mutations to form the overall risk, as has been proposed also for the *CHEK2* 1100delC moderate-penetrance variant (CHEK2 Breast Cancer Case-Control Consortium 2004). The considerable increased risk in mutation carrying families supports the potential for genetic testing of *PALB2* 1592delT in the clinical management of breast cancer families in Finland. As this study focused only on the relatively frequent founder mutation and previous studies have analyzed only 113 breast cancer families from northern Finland for variation in the whole coding region of the gene (Erkko et al. 2007), it is possible that also other rare *PALB2* mutations are present among Finnish breast cancer families, likely with similar frequencies as seen in other populations. Two *PALB2* carriers also harbored mutations in the *BRCA2* gene. Such double mutations have not been identified elsewhere, although one case with both *PALB2* and *BRCA1* mutations has been reported (Pern et al. 2012). These patients did not show any particularly striking phenotype, and the potential effects of double mutations require further investigations. They were diagnosed with breast cancer at the ages of 38 and 46 years, which is slightly younger than the mean age of diagnosis of *BRCA2* mutation carriers of 47 years. No conclusion can be made from the segregation of the variants in these families due to the lack of samples from the family members.

The tumor phenotype of *PALB2* mutation carriers has been reported with a relatively small number of tumors in separate datasets with incomplete conclusions, and has mostly had limited clinical information (Tischkowitz, Xia 2010). The 33 tumors of *PALB2* mutation carriers in this study account for 29% of all *PALB2* carrier breast tumors published so far and an even larger proportion of tumors with information available on clinically relevant tumor characteristics. The comparison of tumor characteristics reported in different studies is challenging as methods and protocols vary between publications. Here, the *PALB2*-related tumors presented properties of aggressive disease with high grade and proliferation status, and were frequently triple negative for ER, PgR, and HER2. These features are largely shared with other studies (Table 4) when the invasive breast tumors described in 14 studies on carriers of 23 different truncating *PALB2* mutations are combined and the numbers compared to those of the *PALB2*-related tumors described in this study. Triple negative tumors, however, are clearly less frequent with borderline significant *P*-values among the *PALB2* mutation carriers in other studies, and ER, PgR, and HER2 negativity is also in general more frequent in our study. Different mutations may have different effects on tumor phenotype and, indeed, none of the other seven tumors of 1592delT mutation carriers described so far showed high expression of HER2 (Erkko et al. 2007). Other variants present in distinct populations may also affect the tumor properties individually or by modifying the effect of a *PALB2* mutation. The collection of the patient material may also be biased and affect the comparison of the studies.

As *PALB2* and *BRCA2* function very closely together, it could have been presumed that their germline loss would result in similar tumor phenotypes. This, however, was not the case here, as the tumors of *PALB2* carriers resembled more closely those of *BRCA1* mutation carriers in many properties. For example both *PALB2* and *BRCA1* tumors were frequently triple negative, whereas *BRCA2*-related tumors were more often Luminal, as has been previously reported (Sorlie et al. 2003, Melchor et al. 2008). Whether other alleles also affect the characteristics of the tumors of *PALB2* mutation carriers remains to be investigated. This would likely require much larger data sets due to the low frequency of the mutations.

Table 4. The frequencies of *PALB2*-related tumors published (Erkko et al. 2007, Foulkes et al. 2007, Tischkowitz et al. 2007, Garcia et al. 2008, Cao et al. 2008, Sluiter et al. 2009, Papi et al. 2010, Balia et al. 2010, Dansonka-Mieszkowska et al. 2010, Hellebrand et al. 2011, Bogdanova et al. 2011, Pern et al. 2012, Teo et al. 2013a, Teo et al. 2013b) compared to the tumors of *PALB2* mutation carriers and non-carriers, wild type (WT), from this study. *P*-values are calculated for the difference between *PALB2* carriers of this study compared to other studies.

Category	***PALB2*** **Other studies**	**%**	***PALB2*** **This Study**	**%**	**WT This Study**	**%**	***P*-value**
Tumor histology							
ca ductale	53	(82.8%)	25	(75.8%)	1526	(67.3%)	0.4068
ca lobulare	8	(12.5%)	3	(9.1%)	481	(21.2%)	ductal vs other
ca medullare	1	(1.6%)	0	(0.0%)	28	(1.2%)	
other	2	(3.1%)	5	(15.2%)	232	(10.2%)	
Grade							
1	3	(6.5%)	3	(9.4%)	574	(26.7%)	0.7859
2	20	(43.5%)	12	(37.5%)	972	(45.2%)	1 and 2 vs 3
3	23	(50.0%)	17	(53.1%)	604	(28.1%)	
ER							
positive	44	(67.7%)	16	(53.3%)	1757	(81.5%)	0.1775
negative	21	(32.3%)	14	(46.7%)	398	(18.5%)	
PgR							
positive	29	(49.2%)	13	(43.3%)	1420	(66.0%)	0.6032
negative	30	(50.8%)	17	(56.7%)	733	(34.0%)	
HER2							
postive	8	(21.6%)	1	(4.3%)	190	(15.1%)	0.1337
negative	29	(78.4%)	21	(95.5%)	1070	(84.9%)	
Subtype							
ER/PgR+/HER2-	17	(48.6%)	9	(40.9%)	900	(73.8%)	0.0499
ER/PgR+HER2+	7	(20.0%)	1	(4.5%)	118	(9.7%)	TripleNeg vs
ER&PgR-HER2+	1	(2.9%)	0	(0.0%)	69	(5.7%)	other
TripleNeg	10	(28.6%)	12	(54.5%)	132	(10.8%)	

The *PALB2* mutation also affected the long-term prognosis of breast cancer patients in familial cases and among patients with HER2 negative tumors. This association was similar to that of patients with a *BRCA2* mutation, which was also found to be an independent prognostic factor in the multivariate analysis. The survival effects related to the mutations of both of these genes became noticeable only after five years of follow-up. Previous studies have mostly focused on shorter-term survival, which might explain why *BRCA2*-related survival effects have not been as strong as the effects described here. Recently, however, the effects of *BRCA2* mutations on breast cancer prognosis have, consistently with our results, been clarified and the mutations indeed seem to associate with worse survival in long-term

follow-up analysis (Goodwin et al. 2012). *BRCA1* mutations did not associate with long-term survival of breast cancer patients, but in the Kaplan-Meier plots, a slight differentiation in the survival rate can be seen at an early state of the follow-up, although this effect was not statistically significant. It remains unlikely that *BRCA1* mutations would play major impact on breast cancer prognosis (Rennert et al. 2007, Bordeleau et al. 2010, Goodwin et al. 2012).

6.2 *PTEN* promoter variants

In the screening of the promoter region of the *PTEN* gene, three low-frequency variants, -903GA, -975GC, and -1026CA, were identified. None of the variants associated with breast cancer risk, although very low risk effects cannot be excluded. The *PTEN* promoter variants associated with the prognosis of breast cancer patients. The -975GC variant also associated with metastasis at diagnosis and -1026CA with higher level of Ki67 proliferation marker. In multivariate survival analysis, carrying a *PTEN* promoter variant approximately doubled the risk of death due breast cancer within 10 years, or developing a distant metastasis or dying of breast cancer within five years from diagnosis. These findings indicate that the *PTEN* promoter variants may have an effect on the tumor progression and metastatic potential. The promoter variants were located in nucleotides evolutionarily conserved in higher mammals, which supports their functionality. None of the promoter variants located at the sites of known transcription factor binding sites on the *PTEN* promoter region (Figure 2), although effects on yet unidentified regulatory mechanisms cannot be excluded.

The most likely function of a promoter variant would be regulatory effects on the gene. No difference in *PTEN* expression level was, however, detected between the tumors of variant carriers and non-carriers in expression microarray analysis, and it is possible that other somatic changes in tumors might have masked the effect. Very strong changes in the *PTEN* expression level would anyway be unlikely in the case of these variants, as effects that dramatic would lead to a PHTS phenotype, such as Cowden's syndrome, in the variant carriers, which clearly was not the case here. Promoter mutations have indeed been shown to cause these diseases (Zhou et al. 2003). The variants detected here may affect the expression levels of *PTEN* on a smaller scale, consistently with the recent model of dosage-dependent tumor suppression of *PTEN* (Carracedo et al. 2011). Indeed, even a subtle decrease in the amount of the PTEN protein have been shown to increase cancer susceptibility in mice, to affect cellular proliferation in mouse mammary tissue, and affect the expression of

proliferation-related genes (Alimonti et al. 2010). As the promoter variants also lay on the 5'UTR of the *PTEN* gene, it is possible that they may affect post-transcriptional regulation of protein synthesis, for example through mechanisms similar to those of eIF4F complex. Indeed some promoter mutations found in patients with Cowden's syndrome affect the expression of the PTEN protein through dysfunctional translation instead of altered transcription by causing large mRNA secondary structure alterations (Teresi et al. 2007).

The breast tumors of the patients carrying a *PTEN* promoter variant compared to matched tumors of non-carriers presented differential expression of 160 genes with patterns similar among -903GA, -975GC, and -1027GA carriers. The functional annotation linked the up-regulated genes to entities of ATP or nucleoside binding and phosphorylation. Down-regulated genes related to entities of DNA binding and transcriptional functions. The strongest down-regulation was seen in the *BAMBI* gene, a regulator of TGF-beta-family signaling (Onichtchouk et al. 1999). Increased expression of *BAMBI* has been detected in ovarian cancer (Pils et al. 2010) and it also predicts aggressive tumor phenotype and prognosis in colorectal cancer (Togo et al. 2008) as well as metastatic potential (Fritzmann et al. 2009). The expression of *BAMBI* is epigenetically silenced in high-grade bladder cancer (Khin et al. 2009). In mice the expression of *BAMBI* is limited to endothelial cells in which it acts as a regulator of angiogenesis (Guillot et al. 2012). The differentially expressed genes identified here, however, represent complex down-stream effects of *PTEN* promoter variants, and as they are measured in developed tumors, other regulatory abnormalities are likely present. Compared to the gene expression signature of somatic *PTEN* loss by Saal et al. (Saal et al. 2007) on the single gene level there was not much overlap. The somatic loss of gene expression of a major tumor suppressor happening at an advanced state of tumor development is, however, a different event from carrying a predisposing germline variant, affecting the tumorigenesis at an early state. The Saal signature genes annotated, nevertheless, to overlapping functional categories, as did the genes in the signature described here.

When the 160 signature genes were used to divide the tumors of 183 patients by clustering analysis based on the overall expression into two groups, an association between the signature and patient survival was revealed. This association was confirmed in independent datasets, indicating that the signature has similar prognostic effects to *PTEN* promoter variants and highlights the biological importance of the signature genes. The prognostic effects of the promoter variants may be carried out through their downstream effects on the signature genes.

When the Saal signature was applied for survival analysis, the clustering of the tumors into two categories was highly correlated between the signatures, emphasizing their similarity and supporting the role the germline promoter variants play on *PTEN*-mediated tumor suppression.

6.3 eIF4E

Previous studies have established eIF4E as prognostic marker in breast cancer, but treatment stratified associations have not so far been investigated, nor survival of post-metastatic patients. One study had looked into the effects of eIF4E expression together with TLK1B (Tousled-like kinase 1B) in breast cancer in doxorubicine-treated patients with a significant effect on survival, but had no non-treated patient group available for comparison (Byrnes et al. 2007). The study here, with 1,085 cases, is thus far the largest and most complete analysis of eIF4E in breast cancer. High expression of eIF4E was associated with characteristics of aggressive breast cancer and also with the triple negative subtype. Here, the most notable survival effect was seen in patients treated with anthracycline-based chemotherapy, suggesting that the patients with high levels of eIF4E in their tumors may have a compromised response to anthracycline. High expression of eIF4E also associated with survival in lymph node-positive and in metastasized breast cancer, suggesting that it has functions in these advanced forms of cancer and emphasizing the role of the protein as a prognostic biomarker with also a potentially predictive value.

The overall picture of the cancer-related targets eIF4E regulates remains unclear (Hsieh, Ruggero 2010). Many such transcripts are known, for instance the MCL-1 (Wendel et al. 2007) protein which has antiapoptotic properties, and when overexpressed, has been linked to high grade of breast cancer and worse prognosis (Ding et al. 2007). Another potential regulatory target of eIF4E is TLK1B, which links DNA damaging agents, such as anthracycline, to eIF4E over expression, as TLK1B functions in the repair of DNA double-strand breaks caused by doxorubicine or radiation (Sunavala-Dossabhoy et al. 2004) and its over expression has been associated with radio resistance (Li et al. 2001). The translation of numerous transcripts is, however, regulated by eIF4E, and to reveal the mechanisms behind results described here, further functional investigations are required. There is also potential to apply eIF4E in cancer treatment by targeting it directly by using, for instance, synthetic nucleotide cap-analogs or antisense oligonucleotides (Malina et al. 2011, Graff et al. 2008, Hsieh, Ruggero 2010).

7 Summary and Conclusions

Since the discovery of the *PALB2* gene in 2006 and its initial identification as a breast cancer susceptibility and Fanconi anemia gene the following year, the gene has been under investigation by several research groups in various populations. The low frequency of mutations has, however, limited the characterization of the tumor phenotype and even more the association analysis of prognostic effects among the mutation carriers. The Finnish founder mutation 1592delT was present in 19 familial (2.0%) and 8 sporadic (0.6%) breast cancer patients and in 2 (0.2%) population controls. This confirmed the risk effect of the mutation and demonstrated a trend of increasing frequency of the mutation with increasing family history of breast cancer. The risk estimation of the *PALB2* mutation will become more precise in the near future when the efforts of an international consortium with large enough sample sets for thorough analyses will be finalized.

When the properties of 33 tumors of the mutation carriers were analyzed, they were associated with higher grade and expression of Ki67 proliferation marker and presented more frequently clinically challenging triple negative subtype. The *PALB2* mutation was also associated with breast cancer survival, particularly in familial patients and in patients with HER2 negative tumors. These features were shared with the *BRCA2* mutation carriers. The results here also indicate *BRCA2* mutation as a prognostic factor, which together with other recent findings (Goodwin et al. 2012), supports preventive measures and extended and more careful follow-up of the patients with *BRCA2* mutations. The genetic testing of *PALB2* mutations in breast cancer families in Finland might be beneficial for the risk assessment and screening of the healthy mutation carriers and also for the management of treatment and follow-up of the affected individuals. The relatively high frequency of the 1592delT mutation in Finland should make the genetic testing cost-efficient together with *BRCA1* and *BRCA2* founder mutations. Further studies are, however, required also to identify other risk variants in the *PALB2* carrier families, as the increase in risk caused by the mutation is unlikely to account for the entire cancer burden in these families.

As a key regulator of activation of the PI3K/AKT pathway and as a major tumor suppressor, *PTEN* is one of the most thoroughly studied human genes. Three low-frequency promoter polymorphisms, -903GA, -975GC, and -1026CA, associated with worse survival of breast cancer patients and presented an expression signature of 160 genes in the tumors of variant carriers compared with matched non-carriers. The signature genes further stratified breast

cancer patients into distinct survival groups in independent datasets, supporting the effects of germline variants on gene expression signatures and on metastatic development in breast cancer. This suggests that the germline *PTEN* promoter variants affect metastatic potential and tumor progression in breast cancer and indicates that they are prognostic markers. To our knowledge these findings are the first clinically relevant associations of germline *PTEN* variants outside hamartomatous polyposis syndromes, although further studies are warranted to validate these findings. The results are in line with the dosage-dependent model of PTEN tumor suppression, according to which even very subtle reduction of the amount of the PTEN protein affects the tumor development. Associations of variants this rare would not have been detected in GWAS on breast cancer survival as they have been powered only to detect stronger effects of more common variants.

Abnormal translation is one of the mechanisms frequently occurring during carcinogenesis. A major regulator of translation of many cancer-related transcripts is the eIF4F complex, which is regulated strongly by the amount of free eIF4E. The expression of eIF4E has previously been associated with prognosis of breast cancer, but the studies have been of very limited size. Here the expression of eIF4E was investigated by thus far the largest and most specifically characterized dataset, revealing associations with multiple characteristics of aggressive breast cancer and with the triple negative subtype. High expression of eIF4E also associated with the survival of breast cancer patients and, in subgroup analysis, a particularly strong effect was identified in patients treated with anthracycline-based chemotherapy. These results support eIF4E as a prognostic marker and emphasize the potential of therapies targeting this protein.

The findings of this study emphasize the potential of germline variants in high and moderate-penetrance susceptibility genes like *BRCA2* and *PALB2*, as well as polymorphic variants like the *PTEN* promoter SNPs, as prognostic markers in breast cancer. The role of eIF4E as a biomarker for aggressive disease with also predictive value might in the future help the assessment of cancer treatments, particularly anthracycline-based treatment. The breast cancer patients with the changes described here might benefit from more rigorous follow-up and from more aggressive treatment.

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